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R E S E A R C H A R T I C L E

Elucidation of *In-Vitro* and *In-Vivo* Cytotoxic Effects of Saffron in C1271 Breast Cancer Cells

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A B S T R A C T

Introduction: Breast cancer is second most common in women and accounts for 23% of all occurring cancers in women. At present, more than 60% of the chemotherapeutic drugs are developed from plants and their derivatives. In the last few years, various medicinal properties of crude saffron extracts have been reported including its inhibitory effects on carcinoma, sarcoma, leukemia, and several other malignant cells *in-vitro.* The present investigation therefore, aimed to study the *in-vitro* and *in-vivo* effects of saffron in C1271 breast cancer cells. **Material and Methods:** Extraction of saffron stigma was done by crystillization method and 80% Ethanol was chosen as the best extraction solvent. For *in-vitro* analysis MTT cytotoxicity assay was carried out. For *in-vivo* analysis female *Swiss albino* mice were grouped into five groups of four animals each and treated with drug control (Doxorubicin), 50mg/kg body wt. crocin, 50% and 80% saffron stigma extract respectively. One group was fed with double distilled water and used as control. **Results:** The results of MTT assay of breast cancer cell line revealed maximum percentage growth inhibition by 60% crocin. The Tukey test of one-way ANOVA revealed significant tumor regression in crocin treatment group *in-vivo* as compared to the tumor control group. The present study suggests saffron as a potent antitumor agent against breast cancer cells.

Keywords: *Crocus sativus*, breast cancer, C1271, cytotoxicity, antitumor activity.

A R T I C L E I N F O

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1. Introduction

Breast cancer continues to be a major health problem for women worldwide. Globally, breast cancer is the most common type of cancer and the leading cause of cancer-related mortality in women. Approximately one-third of women with breast cancer develope metastases and ultimately die from the disease [1]. Breast cancer causes due to various genetic and epigenetic alterations that affect the regulation and function of genes.

Current systemic therapies for breast cancer are often limited by their nonspecific mechanism of action, unwanted toxicities on normal tissues, and short-term efficacy due to the emergence of drug resistance [2]. In addition to the synthetic drugs, immense use of natural products and its derivatives in the development of anticancer drugs are increasing all over the world because of lesser side effects as compared to synthetic drugs [3-5]. As more than 60% of the chemotherapeutic drugs are developed from plants and their derivatives, medicinal plants have been potential sources of natural products exhibiting anti-proliferation and anti-metastatic properties [6].

Saffron (*Crocus sativus L)* belongs to family Iridaceae and it is considered as one of the worthiest perennial flowers with a violet color and usually 3 golden petal stigmas. Saffron, the dry stigmas of the plant *Crocus sativus* L., belongs to the Iridaceae family and principally grows in Iran and Spain [7]. Although it is currently used as a spice and food colorant, however, traditional medicine has used saffron in the treatment of numerous illnesses including cough, colic, insomnia, chronic uterine hemorrhage, cardiovascular disorders and tumors [8]. In the last few years, the antitumor properties of crude saffron extracts, both *in vitro* and *in vivo*, have been demonstrated. It has exhibited a dose dependent inhibitory effect on carcinoma, sarcoma, leukemia, and several other malignant cells *in vitro.* Saffron increased life span of treated tumor-bearing mice compared to untreated animals by 45-120%. The present study therefore, explored the *in-vitro* and *in-vivo* cytotoxic effect of saffron extract using C1271 breast cancer cell line as a prominent model system.

2. Materials and Methods

Collection and authentication of plant

Crocus sativus plant was collected from District Pulwama of Jammu & Kashmir. The plant was identified by a Senior Taxonomist at University of Kashmir on the basis of its organoleptic and microscopic examination. Plant Herbarium was submitted at University of Kashmir with Voucher Numbers. 2458, 2459-A, 2459-B, 2459-C. Saffron stigma was collected and shade dried before extraction.

Extraction of Saffron

Crystallization method was used to extract the saffron stigma. For saffron bulb Soxhlet apparatus extraction method was used. 80% ethanol was chosen as the best extraction solvent. Saffron stigma (12gm) was suspended in 25ml ethanol at $0^{\circ}C$ and shaken by vortex for 2 minutes after centrifugation for 10 minutes at 4000rpm the supernatant was separated. Twenty five millilitres of 80% ethanol were added to sediment and extraction was repeated again. The total volume of solvent consumption in extraction process was 200ml. The resulting solution was kept in a thick walled glass container for one week at $-5^{\circ}C$ in darkness. The container was sealed during that period. The obtained crystals were separated from the solution and washed with acetone to remove remaining water.

Procurement and maintenance of cells

Mice cancer cell line C1271 was procured from National Center for Cell Science (NCCS) Pune. Cells were grown in tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) with 2mM glutamine, pH 7.4, supplemented with 10% fetal calf serum, 100 µg/ml streptomycin and 100 units/ml penicillin) in a carbon dioxide incubator (37°C, 5% CO_2, 90% RH). The cells at subconfluent stage were harvested from the flask by treatment with trypsin [0.05% in PBS (pH 7.4) containing 0.02% EDTA]. Cells with viability of more than 98% as determined by trypan blue exclusion, were used for determination of cytotoxicity. The cell suspension of 1x105 cells/ml was prepared in complete growth medium.

MTT-cytotoxicity assay

Cytotoxic effect of all the extracts were determined as per Razzaq et al., 2011[9]**.** 96-well culture plates were seeded at 10,000 cells per well and incubated in CO_2 incubator (Heracell, Heraceus Germany) for 24-hours. Experiment was carried out by adding 50µl of prepared concentrations of all the extracts and stock solutions of all the extracts 1mg/1ml) diluted to 20, 40, 60, 80, 100µg/ml) in triplicate into appropriate wells and reincubated for 72-hours at the same condition. Media alone was taken as Blank and media plus cells were treated as control. MTT solution was prepared at 5mg/ml in PBS and was filtered through a 0.2µm filterate. 20µl MTT solution was added to each well and mixed by tapping gently on the side of the tray and incubated at $37^{0}C$ for 4-hours. 100µl of old media containing MTT was then gently replaced by 100µl DMSO into each well and to dissolve the formazane by pipetting several times. The absorbance was measured on ELISA plate reader (STAFFA diagnostic Pvt. Ltd. Chennai-India), at a test wavelength of 492nm and a reference wavelength of 630nm. Concentration of each extracts was analysed in 5-wells and experiment was done in triplicate. The percentage growth inhibition was assessed taking percentage cell growth into consideration. The treatment showing highest percentage growth was considered to have lowest percentage growth inhibition.

Experimental animals and dosage

Adult female *Swiss albino* mice aged 6-8 weeks with an average weight of 30gms were obtained from animal house of Jawaharlal Nehru Cancer Hospital & RC, Bhopal. The animals were housed in standard polypropylene cages and maintained in air conditioned animal house ($20\text{-}25^{0}C$ relative humidity 70-75%) in a 12-hour light-dark cycle. The animals were fed on a standard laboratory diet and water *ad-libitum*. The studies were done with the prior permission and approval of CPCSEA and norms of IAEC Ref.No.500/01/a/CPCSEA/2001. Breast tumors were

induced in the mice by injecting calculated number of C1271 cells intradermally and the mice with palpable tumors were used as *in-vivo* tumor models. The experimental animals were divided into five groups of four animals each. Two groups were given 50% and 80% dosage of Saffron stigma extract. One group was given active constituent Crocin dosage, one group was used as tumor control and another group was given standard drug (Doxorubicin) dosage and used as Standard drug control.

Statistical analysis

Statistical analysis was carried out using Origin 8.0 Software. Student's t test was performed on the means of percentage growth of cells in the *in-vitro* analysis of saffron dosages on cancer cells. One-way ANOVA was carried out to assess the tumor regression in various treatment groups *in-vivo.*

3. Results and Discussion

In-vitro cytotxicity results

The results of MTT assay of breast cancer cell line revealed maximum percentage growth inhibition by 60% crocin. In ethanolic stigma extract group, 40% concentration showed the maximum percentage inhibition, while 80% concentration showed the minimum percentage growth inhibition. 60% and 100% concentrations of ethanolic stigma extract were found to have prominent percentage growth inhibition. However 80% ethanolic stigma extract concentration was found to have lesser percentage growth inhibition as compared to Doxorubicin. All the concentrations (20%, 40%, 60%, 80%, and 100%) of ethanolic stigma extract were found to have lower percentage growth inhibition as compared to Methotrexate.

In methanolic stigma extract group, 20% concentration showed the maximum percentage inhibition, while 60% concentration showed minimum percentage inhibition against breast cancer cell line. 20% and 40% concentrations of methanolic stigma extract were found to have better percentage growth inhibition as compared to Doxorubicin. However, 60%, 80% and 100% concentrations were found to have lower percentage growth inhibition as compared to Doxorubicin. Besides, all the concentrations (20%, 40%, 60% and 100%) of methanolic stigma extract exhibited statistically lower percentage inhibition as compared to Methotrexate.

In saffron bulb extract group, 80% dosage proved to be the best in terms of percentage growth inhibition, while as 40% dosage showed the minimum percentage inhibition. As compared to Doxorubicin, 20%, 60%, 80% and 100% concentrations of saffron bulb produced prominent percentage growth inhibition, while 40% concentration produced lesser percentage growth inhibition. All the concentrations of saffron bulb extract were found to produce lesser percentage growth inhibition as compared to Methatrexate.

Comparing 20% concentration of ethanol stigma, methanol stigma, saffron bulb and crocin, 20% methanol stigma produced the maximum percentage growth inhibition, while crocin produced minimum percentage growth inhibition. Comparing 40% concentrations of ethanol stigma, methanol stigma, saffron bulb and crocin, 40% ethanol stigma produced maximum percentage inhibition, while 40% saffron bulb produced minimum percentage growth inhibition.

Comparing 60% concentrations of ethanol stigma, methanol stigma, saffron bulb and crocin, 60% crocin produced maximum percentage growth inhibition, while as 60% methanol stigma produced minimum percentage growth inhibition. Comparing 80% concentrations of ethanol stigma, methanol stigma, saffron bulb and crocin, saffron bulb produced maximum percentage growth inhibition, while ethanol stigma produced minimum percentage growth inhibition.

Comparing 100% concentrations of ethanol stigma, methanol stigma, saffron bulb and crocin, 100% ethanol stigma produced maximum percentage growth inhibition and crocin produced minimum percentage growth inhibition.

In crocin group, 60% dosage showed the maximum percentage inhibition. Comparing it to Doxorubicin and Methotrexate, the percentage inhibition was statistically lower. The results of the MTT assay of breast cancer cell line are tabulated as Table 1.

Table 1: Showing mean absorbance and percentage growth in various treatment groups of saffron in breast cancer cell line.

Groups	Absorbance (Mean± SEM)	Percentage Growth
Cell Only	0.93±0.33	
DDW	2.19±0.20	402.86
Doxorubicin	2.42±0.09	202.82
20% EtOH Stigma	2.33±0.11	249.94
40% EtOH Stigma	2.20±0.51	220.55*
60% EtOH Stigma	2.23±0.14	225.18*
80% EtOH Stigma	2.89±0.22	310.31
100% EtOH Stigma	2.8±0.11	244.57
20% MeOH Stigma	2.35±0.47	229.34*
40% MeOH Stigma	2.41±0.19	258.53
60% MeOH Stigma	2.80±0.13	311.06
80% MeOH Stigma	2.62±0.16	280.98
100% MeOH Stigma	2.59±0.23	278.3
20% Saffron Bulb	2.20±0.18	237.05*
40% Saffron Bulb	2.62±0.08	281.74
60% Saffron Bulb	2.24±0.02	241.13*
80% Saffron Bulb	2.19±0.31	227.91*
100% Saffron Bulb	2.34±0.11	251.77
20% Crocin	2.84±0.12	305.26
40% Crocin	2.48±0.20	266.7
60% Crocin	1.75±0.35	210.93*
80% Crocin	2.19±0.15	235.76*
100% Crocin	3.41±0.03	366.8
Methatrexate	0.711±0.098	76.36

* Comparable percentage growth inhibition as compared to Doxorubicin

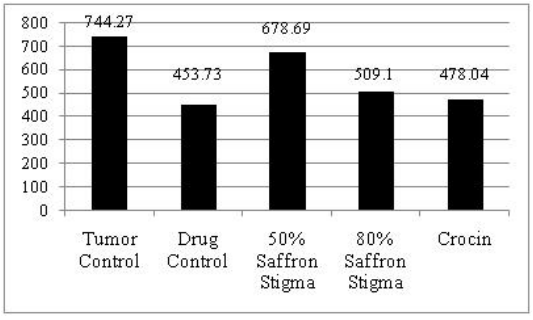
***In-vivo* anti-tumor activity using breast cancer tumor models:** The results of *in-vivo* anti-tumor activity using breast cancer tumor models (Figure 1) revealed maximum tumor regression in animal group treated with crocin. The Tukey test of one-way ANOVA revealed significant tumor regression in crocin treatment group as compared to the tumor control group. Besides the mean reduction in the tumor volume of crocin treated group was significantly higher as compared to 50% and 80% saffron stigma groups. Crocin treatment produced comparable tumor regression as that of the standard drug. In 80% saffron stigma treatment group, the mean tumor was found to be statistically reduced as compared to tumor control and 50% saffron stigma group.

Figure 1: Breast cancer tumor bearing Swiss albino mice used in the present study.

Table 2: Showing the results of *In-vivo* anti tumor activity in various treatment groups of breast cancer

Groups	Tumor Volume (Mean± SEM)
Tumor Control	744.27±6.94
Drug Control (Doxorubicin)	453.73±1.80
50% Saffron Stigma	678.69±2.54
80% Saffron Stigma	509.10±1.36
Crocin	478.04±1.24*

*Comparable anti-tumor activity against breast cancer *in-vivo* as that of the drug control.



Graph 1: Showing mean tumor volume in various treatment groups of *in-vivo* antitumor study.

Discussion

***In-vitro* antitumor activity**

The results of MTT cytotoxicity assay of breast cancer cell line revealed maximum percentage growth inhibition by 60% crocin. In ethanolic stigma extract group, 40% concentration showed the maximum percentage inhibition, while 80% concentration showed the minimum percentage growth inhibition. 60% and 100% concentrations of ethanolic stigma extract were found to have prominent percentage growth inhibition. However, 80% ethanolic stigma extract concentration was found to have lesser percentage growth inhibition as compared to Doxorubicin. In methanolic stigma extract group, 20% concentration showed the maximum percentage inhibition, while 20% and 40% concentrations of methanolic stigma extract were found to have comparable percentage growth inhibition as that of Doxorubicin. In saffron bulb extract group, 80% dosage proved to be the best in terms of percentage growth inhibition, while as compared to Doxorubicin, 20%, 60%, 80% and 100% concentrations of saffron bulb produced prominent percentage growth inhibition. In crocin group, 60% dosage showed the maximum percentage inhibition. Comparing it to Doxorubicin and Methotrexate, the percentage inhibition was statistically lower. These results suggest that saffron extract and its active constituent crocin has a remarkable cytotoxicity activity *in-vitro* against breast cancer line C1271. This is in accordance with a study which investigated the effect of extracts of the styles of *C. sativus*, *C. boryi* ssp. *tournefortii*, *C. boryi* ssp. *boryi* and *C. niveus* on breast cancer (MCF-7 and MDA-MB-231) cell proliferation. Incubation of MCF-7 and MDA-MB-231 breast cancer cells for 48h with different concentrations of all four Crocus style extracts showed a dose-dependent inhibitory effect on cell proliferation measured by the MTT assay [10]. In an Iranian study, the cytotoxic and apoptogenic effects of saffron in MCF-7 cells as an *in-vitro* model for breast cancer study were investigated and the role of caspases were studied using the pan-caspase inhibitor, z-VAD-fmk. Saffron extract decreased cell viability in MCF-7 cells as a concentration and time-dependent manner. Saffron has also been found to induce apoptosis in MCF-7 cells in which apoptosis was dependent on caspase activation [11].

Previous studies have also evidenced cytotoxic activity of dimethyl-crocetin, crocetin, and crocin in human chronic myelogenous leukemia K562 and promyelocytic leukemia HL-60 cells [12]**.** The cytotoxic activity of crocin has also been demonstrated with rat adenocarcinoma DHD/K12-PROb cells and human colon adenocarcinoma HT-29 cells with ID50 (50% inhibitory dose) values of 0.4 and 1.0 mM, respectively and with HeLa cells[13].

Jagadeeswaran *et al.* have observed that an inhibition of proliferation of rhabdomyosarcoma cells was induced by crocetin, whereas Tarantilis *et al.* have reported that crocetin displayed a strong antiproliferative action on HL-60 and K562 cells [14,12]. Previous studies have also shown that orally ingested crocins could not act as bioactive molecules by themselves *in vivo* except in the gastrointestinal tract since they are hydrolyzed to crocetin before being incorporated into the blood circulation [15].

Therefore, the fact that crocetin is active is of great importance. On the other hand, Escribano *et al.* have reported that crocetin has no cytotoxic effect in HeLa cells [16]**.** The reported discrepancy could be due to the differences in cell lines and culture conditions used and different mechanisms of action.

The cytotoxic effects of saffron in our study are well supported by the findings of Abdullaev *et al.* who have shown that crocetin had a dose-dependent inhibitory effect on the DNA, RNA and protein synthesis of different human malignant cells (HeLa, A-204, HepG-2, CCD), but no significant effect on colony formation [17]. The cytotoxic effects of saffron can also be attributed to the anti-angiogenesis effect of *Crocus sativus* L. extract on matrix metalloproteinase gene (MMP) activities in human breast carcinoma cells which was evaluated and the data showed inhibitory effect of saffron at concentrations of 100 to 800µg/ml on MMP gene expression in comparison with control group. Results indicated decrease in the expression of MMP compared with controls revealing induction of inhibitory effects of saffron on angiogenesis [18]**.**

A study using saffron extract as an adjuvant to chemotherapy drug Paclitaxel revealed that ethanol and aqueous extract of saffron and Paclitaxel have significant anticancer properties against breast Cancer cells. It was found that the aqueous and ethanol extracts of saffron can considerably increase cytotoxicity in cancer cells induced by Paclitaxel while the aqueous extract of saffron showed better results [19].

The underlying mechanisms of antiproliferative activity of saffron and its constituents are largely unknown. Earlier studies on the effect of saffron constituents on other cancer cells have associated their antiproliferative effect with the inhibitory effect on cellular DNA and RNA synthesis and interaction with topoisomerase II, an enzyme involved in cellular DNA protein interaction [17,7]**.** Another suggested mechanism is the metabolic conversion of naturally occurring carotenoids to retinoids, even though it has been reported that this is not a prerequisite for anticancer activity [7,20]. Escribano *et al.,* 1996 have described various changes in the morphology of treated cells suggestive of apoptosis, while another proposed mechanism is the inhibitory effect of these compounds on free radical chain reactions [20,8].

In-vivo anti-tumor activity

The results of *in-vivo* anti-tumor activity using breast cancer tumor models revealed maximum tumor regression in animal group treated with crocin. The Tukey test of one-way ANOVA revealed significant tumor regression in crocin treatment group as compared to the tumor control group. Besides the mean reduction in the tumor volume of crocin treated group was significantly higher as compared to 50% and 80% saffron stigma groups. In 80% saffron stigma treatment group, the mean tumor was found to be statistically reduced as compared to drug control and 50% saffron stigma group. The results of our *in-vivo* anti-tumor activity were in concordance with that of our *in-vitro* MTT cytotoxicity assay of breast cancer cell line. Although, there is limited data available in the literature on the *in-vivo* anti-tumor activity of saffron and its constituents against breast cancer, ample evidence suggests that saffron extract is a potent antiproliferative agent against breast cancer cells *in-vitro* [10,11,18].

4. Conclusion

The results of the present investigation suggest that saffron extract produces remarkable cytotoxic effects against breast cancer cells *in-vitro* as well as *in-vivo*. It may be postulated that the saffron extract would be helpful in pharmacological applications in treatment of breast cancer. The study will also help to investigate further in elucidating the mechanism involved by active components such as crocin in anticancer activity.

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