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Biological evaluation of saffron *Crocus Sativus* L extracts from Jammu and Kashmir

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Abstract
Saffron, the most expensive spice in the world, and is derived from dried stigma of the saffron flower and is having very high medicinal potential due to presence of some bioactive compounds like crocin, picrocrocin and safranal. In present study bioevaluation studies of different samples of *Crocus sativus* L. collected from different places of Jammu and Kashmir were done in order to estimate the efficacy of different extracts against different cancer cell lines. The bioevaluation studies were carried out by studying the antiproliferative and antioxidant potential of methanolic extracts of different samples of saffron. Saffron extracts showed high antiproliferative activities when used against Caco-2 and A-549 cancer cell lines. Also high antioxidative potential was observed in saffron extracts which was revealed through MTT and DPPH assays. Thus present study clearly deciphers the pharmacological potential of *crocus sativus* L.

Keywords: *crocus sativus*, MTT assay**,** DPPH assay

1. Introduction
Crocus sativus L. is an autumn-flowering geophytes extensively grown in the Mediterranean basin and Near East since the Late Bronze Age [1]. Saffron, the dried red stigmas of *C. sativus*, has been used as flavouring and colouring agent since then and is currently considered the world's most expensive spice. The major components of saffron are the apocarotenoids cis- and trans-crocins, picrocrocin (-D'Glucopyranoside of hydroxyl-_-cyclocitral) and its degradation product, the odour-active safranal [2]. Many studies have demonstrated that the genotypic diversity of *C. sativus* is extremely low [3, 4]. This limited genetic diversity in saffron is attributed to its asexual propagation, followed by successive selection during breeding efforts [3]. Most of the researchers reveal that saffron is monomorphic in nature by using RAPD, SSR and ISSR markers. But still some researchers [5] believe that RAPD markers can be used for identifying the variation within these monomorphic genotypes. PCR-based approaches are in demand because of their simplicity and also because they have shown promise in crop improvement of a large number of crops. Genetic diversity and relationships among species or populations are important topics in genetics and plant breeding. Since saffron is generally monomorphic at morphological level, there is an urgent need to identify the variation at molecular level which can be further exploited for improvement of this crop. Even though qualitative traits of saffron are indeed influenced by sowing time [6] and environmental conditions [7], only few and fragmentary information correlating genetic and biochemical traits is available to date. The discovery of genetic differences in saffron would mean a new way for its improvement, and eventually the possibility to link some particular genetic traits with morphological and biochemical features. Therefore, in order to explore the variability of morphological and qualitative traits among selected saffron clones, molecular evaluation, both at genomic and expression level, was done to identify saffron clones with higher variability in respect to improved quality.
The uses of saffron dates back in to ancient Egypt and Rome, wherein it was used for perfumery, spice and dying purposes. But the uses of saffron in traditional medicine for treatment of illnesses, including cough, colic, insomnia, chronic uterine hemorrhage, cardiovascular disorders and tumors have been reported. [8] Potential uses of saffron extract as anticancer and antitumor properties and cytotoxic effect has been studied in the breast cancer cell lines, MCF-7 [9]. There are also some reports on antiproliferation of lung cancer cell lines by saffron extracts. [10] However, there is no evidence on the therapeutic effects of saffron extracts from Jammu and Kashmir region on cancer cell line. Therefore, the aim of the present study was to assess the potential cytotoxic and antiproliferative effects of saffron (*C. sativus* L.) in human lung and colon cancer cell lines.

Materials and methods
Collection of samples
The samples were collected from different ecogeographical zones of Jammu and Kashmir covering high altitude regions like Kishtwar, Pulwama, Budgam and Anantnag (Table 1) and voucher specimens were deposited in the Herbarium of the institute under voucher specimens (No.I302/2012-I105/2013).

Table 1: Summary for the tested samples of *saffron*

No.	Code	Samples	Sources	Collection date
1	SF-6	*Crocus sativus*	Sambora, Pampore	Oct-nov. 2013
2	CF-10	*Crocus sativus*	Charisharief, Budgam	Oct-nov. 2013
3	NP-2	*Crocus sativus*	Nehama, Pulwama	Oct-nov. 2013
4	BD-1	*Crocus sativus*	Nagam, Budgam	Oct-nov. 2013
5	K-1	*Crocus sativus*	Puchel, Kishtwar	Oct-nov. 2013
6	K-2	*Crocus sativus*	Berwar, Kishtwar	Oct-nov. 2013
7	SF-11	*Crocus sativus*	Duru, Anantnag	Oct-nov. 2013
8	NP-1	*Crocus sativus*	Patlibal, Pampore	Oct-nov. 2013
9	SF-5	*Crocus sativus*	Wolarhama Anantnag	Oct-nov. 2013
10	SF-1	*Crocus sativus*	Parigam, Pulwama	Oct-nov. 2013

Extraction of saffron for bioevaluation

saffron stigma were extracted with methanol (100 mL) in a microcentrifuge tube for 5 min on ice. Tris-HCl (50 mM, pH 7.5; containing 1 M NaCl) was then added (100 mL) and a incubated for 10 min on ice. The precipitate was collected by centrifugation at 3,000g for 5 min at 4ºC. The pellet was then reground in acetone (400 mL) and incubated on ice for 10min. The mixture was centrifuged at 3,000rpm for 5 min at 4ºC. This step was repeated until no color was detected in the pellet. The supernatants were pooled and evaporated and the dried residues were stored at -80ºC until bioevaluation analysis.

Cell lines and cell culture conditions

Caco-2 Colon adenocarcinoma cell line and A549 lung cancer cell line was kindly provided by Hybridoma Laboratory, National Institute of Immunology, India. Cells were grown in DMEM (Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA) under standard culture conditions at 37% in 5% CO_2 in a humidified incubator. 2×10^4 cells were plated per well in a 96 well plate. The media was also supplemented with 250 IU/ml penicillin (invitrogen, USA)

Proliferation assay: The effect of methanolic extracts of different samples of saffron on Caco2 and A549 cells was evaluated using MTT cell proliferation assay. The assay is based on the ability of mitochondrial succinate-tetrazolium reductase system to convert yellow tetrazolium salt MTT (sigma Aldrich, USA) to purple formazan dye that reflects the cell viability. Cell suspension (200ul) containing 2×10^4 cells per well was seeded into a 96 well microtiter plate. After 24 hours of seeding, different concentration of compound dissolved in DMSO (400µg, 300µg, 200µg, 100µg) were added. Controls consisted of either cells alone or cells treated with DMSO (solvent control). MTT solution was added to the cells at 0.1mg/ml concentration followed by incubation for 4h in 37ºC in dark. The supernatant was removed and an equal volume of DMSO was added to dissolve the formazan crystals. The absorbance was measured at 565 nm (EPOCH Microplate Reader, Bio-Tek Instruments, USA).

DPPH Assay: In this assay, free radical scavenging activity of crude methanolic extracts of saffron samples was determined from the bleaching of purple colored methanolic solution of DPPH. The assay comprised of 1 mg/ml of DPPH in presence and absence of test material. The plate was then incubated at 37 ^{0}C for 1 h. After incubation, absorbance was read at 515 nm. From the absorbance, the free radical scavenging activity of each crude methanolic extract relative to that of ascorbic acid was computed using the following:

$$\frac{\text{OD Control - OD Test}}{\text{OD Control}} \text{X } 100$$

Discoloration of DPPH was taken as the indicator of antioxidant activity.

Results and Discussion: Antioxidant activity of saffron extracts: The DPPH method is a simple, practical and sensitive assay which has been widely used to detect active antioxidants with scavenging capacity even in low concentration. [15] The ability of antioxidants to react with DPPH which is a stable free radical and its conversion to α, α-diphenyl-β-picryl hydrazine is expressed in % DPPH inhibition. DPPH by accepting an electron loses its color and changes from purple to yellow. Discoloration degree indicates that the antioxidants possess scavenging potentials. Since DPPH is a stable free radical and does not dimerize as happens with most free radicals, the absorbance diminution depends linearly on the antioxidant concentration. [16-17]. DPPH assay results revealed the antioxidant activities of different extracts of saffron stigmas. The free radical scavenging activity (DPPH) assay indicated a steady increase in the free radical scavenging activity by all the extracts in dose dependent manner (Fig 1). Free radical scavenging percentage ranged from 49-75.66% among all the ten extracts. Maximum free radical scavenging activity (75.66%) was observed in NP-1 extract followed by 74.67% and 73% in SF-8 and NP-2 extracts respectively. Since ascorbic acid showed 84.03% inhibition therefore the scavenging potential of extracts from NP-1, SF-8 & NP-2 are significantly high. Minimum scavenging potential (49%) was observed in CF-10 extract. Free radical scavenging ability of saffron extracts observed in present study was in accordance with previous findings. [3, 18, 19] Their findings also revealed that saffron extracts have high free radical scavenging potential which can significantly contribute to its antioxidative properties. Thus saffron extracts particularly those having higher free radical scavenging potential can have potential chemo preventive roles.

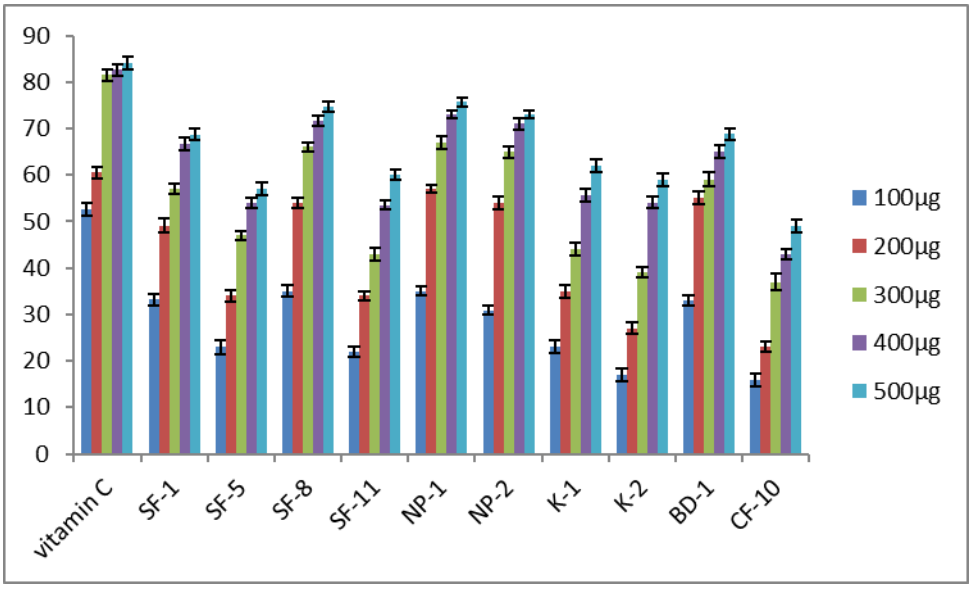

Fig 1: Antioxidant activities relative to ascorbic acid and the crude methanolic extracts of different saffron samples using the DPPH(1,1-diphenyl-2-picrylhydrazyl) assay. Each value represents the mean of three trials with three replicates per fraction per trial.

Effect of saffron extracts on cell viability

In order to evaluate the effect of the different saffron extracts on the growth of human lung and colon cancer cells, the cells were incubated for 48 h with different concentrations of the saffron extract (100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml and 500µg/ml) and their growth inhibitory effects were compared. The impact of the saffron extract on cell viability was quantitated by the MTT assay. The methanolic saffron extract showed significantly high growth inhibitory effects on both the cancer cell lines in a concentration dependent manner (Fig 2 & Fig 3). After 48 h, maximum anti-proliferative activity of saffron was observed on lung cancer cell lines than on colon cancer cell lines in all concentrations. The average ant-proliferative activity of all the ten extracts at 500µg/ml concentration on lung cancer cell line A549 was 70% while on colon cancer cell line Caco-2 average anti-proliferative activity of all extracts was 54%. Although there is significantly high anti-proliferative activity of saffron extracts on colon cancer cell line also but marginally lower than on lung cancer cell line. Therefore our results reveal the potential of saffron extracts for cancer treatment in general and lung cancer in particular. As for as the extracts from different saffron selections is concerned, extract from selection NP-1- was found to be most effective in anti-proliferation (83%) of lung cancer cell line followed by NP-2 (82.16%) and SF-8 (80.56%). Antiproliferative activity on colon cancer cell line was found highest (65.34%) in SF-1 followed by 65% and 61.45% in NP-1 and NP-2 respectively. Therefore our results showed that extracts from NP-1, NP-2, SF-8 and SF-1 have very high potential for controlling lung and colon cancer as revealed by their significantly higher anti-proliferative activities against lung cancer cell line (A-549) and colon cancer cell line (Caco-2). A number of *in vivo* and in vitro experiments indicate that saffron stigma has the potential to reduce the risk of developing several types of cancer. The saffron plant has been shown to be a source of bioactive compounds with cytotoxic, antitumoural, chemo preventive, ant mutagenic and immuno-stimulating properties. Crocin and safranal, the major carotenoid components of saffron stigma, demonstrated antitumor properties, promoting tumor growth inhibition and increasing the life-span of treated tumor-bearing animals. These apocarotenoids do not have any side effects on human being if consumed on large scale. They are regarded as safe bioactive compounds which are naturally occurring in saffron stigma. Crocins and crocetins (the deglycosylated forms) were also found to be potent inhibitors of carcinogenesis as well as attenuators of the toxicity of some anticancer agents. [20] They have an inhibitory effect on the intracellular nucleic acid and protein synthesis in malignant cells as well as on protein kinase C (PKC) and prorooncogene in INNIH/3T3 cells, which is most likely due to their antioxidant activity. [21] Role of saffron extracts in antiproliferation of colon cancer cell lines has been reported [22]. Our data showed that the saffron extract has a higher cytotoxic activity against lung and colon cancer cell lines and supports increasing evidence that naturally occurring saffron extract may have an important role in cancer chemoprevention. Although the results of cell toxicity did not correlate with the actual crocin and safranal contents but the activities shown by all the extracts have significantly higher amount of these apocarotenoids. The activity may be due to presence of group of bioactive compounds among them apocarotenoids are also important. Further studies need to be done for elucidating the role of other constituents and the impact of apocarotenoids in cell toxicity and cancer treatment. Considering the popularity of herbal use in cancer patients, saffron and especially its active ingredients should be investigated further as a viable option in the treatment of colorectal and lung cancer.

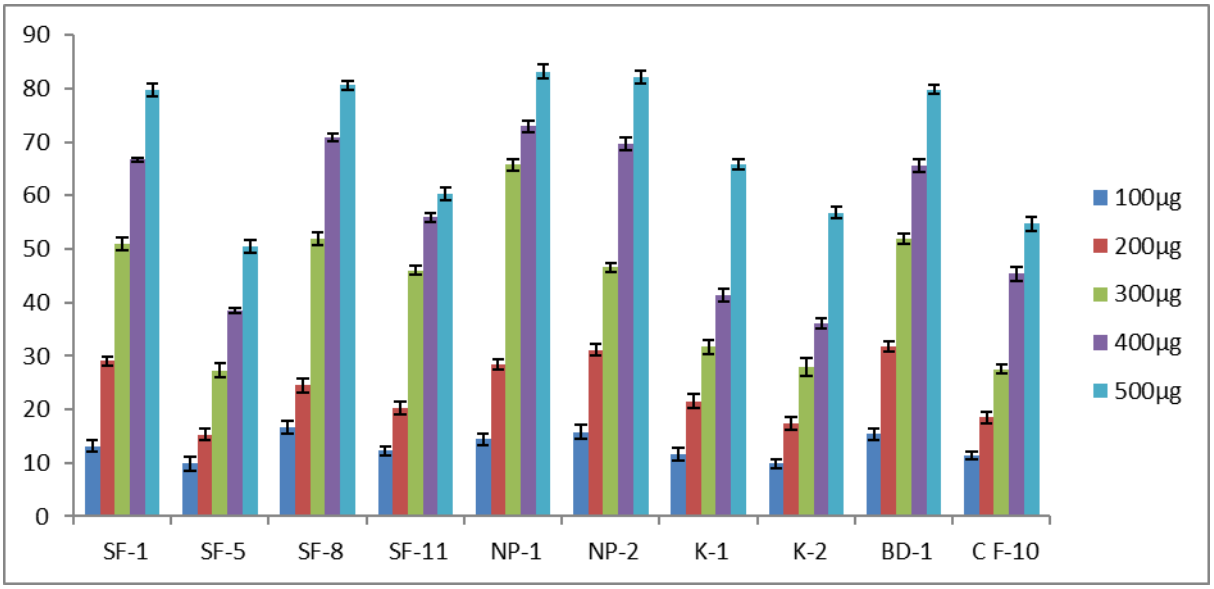

Fig 2: Antiproliferative activity of methanolic extracts of different samples of saffron against A-549 cell line after 48 hour of treatment

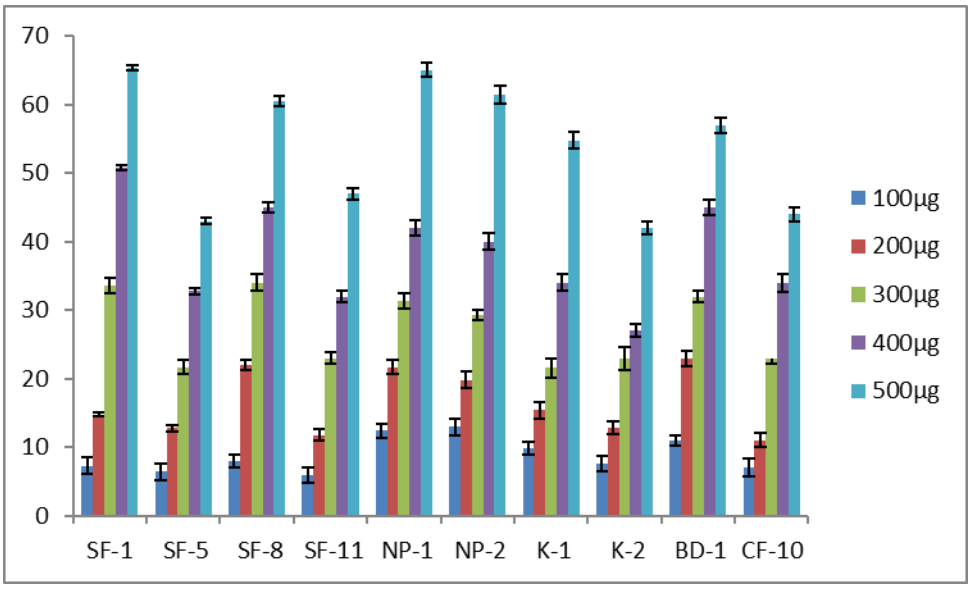

Fig 3: Antiproliferative activity of methanolic extracts of different samples of saffron against caco-2 cell line after 48 hour of treatment.

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