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Use of in vitro assays to assess the potential antigenotoxic and cytotoxic effects of saffron (*Crocus sativus* L.)

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Abstract

Saffron is harvested from the dried, dark red stigmas of *Crocus sativus L.* flowers. It is used as a spice for flavoring and coloring food and as a perfume. It is often used for treating several diseases. We assessed the antimutagenic, comutagenic and cytotoxic effects of saffron and its main ingredients using the Ames/*Salmonella* test system, two well known mutagens (BP, 2AA), the in vitro colony formation assay and four different cultured human normal (CCD-18Lu) and malignant (HeLa, A-204 and HepG2) cells. When only using the TA98 strain in the Ames/*Salmonella* test system, saffron showed non-mutagenic, as well as non-antimutagenic activity against BP-induced mutagenicity, and demonstrated a dose-dependent co-mutagenic effect on 2-AA-induced mutagenicity. The saffron component responsible for this unusual comutagenic effect was safranal. In the in vitro colony formation test system, saffron displayed a dose-dependent inhibitory effect only against human malignant cells. All isolated carotenoid ingredients of saffron demonstrated cytotoxic activity against in vitro tumor cells. Saffron crocin derivatives possessed a stronger inhibitory effect on tumor cell colony formation. Overall, these results suggest that saffron itself, as well as its carotenoid components might be used as potential cancer chemopreventive agents.

Keywords: Saffron *Crocus sativus*; Cytotoxicity; Mutagenicity; Co-mutagenicity

1. Introduction

In view of the renewed interest in chemopreventive plant agents, based on evidence from epidemiological and in vivo and in vitro experimental studies indicating that natural products might protect against the development of various diseases, including cancer, it is important to study the antigenotoxic and cytotoxic effects of saffron (Abdullaev, 2001). Since ancient times, saffron which is harvested from the dried, dark red stigmas of *Crocus sativus* L. flowers, has not only been used as a spice for flavoring and coloring food and as a perfume, but also for treating several diseases. Recent data show that the saffron extract and its components possess anticarcinogenic (inhibition of chemical carcinogenesis) and antitumor (inhibition of tumor growth) in vivo and in vitro activities (Nair et al., 1995; Abdullaev and Frenkel, 1999; Winterhalter and Straubinger, 2000, Pemkumar et al., 2001; Abdullaev, 2002).

Several studies have shown cells to be sensitive to saffron and its components. The differences in sensitivity to the effect of saffron and its main ingredients in normal and malignant cells (Salomi et al., 1991; Nair et al., 1991a,b, 1992, 1994; Tarantilis et al., 1994, Abdullaev and Frenkel, 1992a,b; Abdullaev, 1994, 2002; Chang et al., 1996; Escribano et al., 1996, 1999a–c, 2000; el Daly, 1998; García-Olmo et al., 1999; Molnar et al., 2000) could be due to the existence of distinct cell surface receptors, intracellular retention transport, differences in the uptake of certain drugs or in the methods

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used for the extraction and assessment of toxicity. It was also demonstrated that the saffron extract inhibited cellular nucleic acid synthesis and had no effect on protein synthesis in tumor cells (Abdullaev and Frenkel, 1992a,b; Nair et al., 1995). Interestingly, there was a stimulatory or supporting effect of the saffron extract on the non-specific proliferation of lymphocytes in vitro and on colony formation of normal human lung cells (Nair et al., 1992; Abdullaev et al., 1992a,b).

Characteristic compounds of saffron include crocin, safranal, picrocrocin, crocetin and β-carotene (Tarantilis et al., 1994; Escribano et al., 1996). It was shown that these saffron ingredients inhibited different types of tumor cell growth, with crocetin having no effect on colony formation of tumor cells, although it had a dose-dependent inhibitory effect on DNA, RNA and protein synthesis of different human malignant cells (Abdullaev, 1994; Escribano et al., 1996). It was recently shown that a novel glucoconjugate, isolated from saffron corms and calluses possessed cytotoxic activity against tumor cells (Escribano et al., 2000). Previously, a study using the Ames/*Salmonella* assay and sodium azide as a mutagen, indicated that crocin and dimethyl-crocetin, isolated from saffron, were non-mutagenic, non-antimutagenic and non-comutagenic (Nair et al., 1995). Another study reported that the saffron extract itself was also non-mutagenic (Rockwell et al., 1979). The aim of the present study was to assess the potential genotoxic and cytotoxic effects of saffron (*Crocus sativus* L.) using different in vitro assays.

2. Materials and methods

2.1. Chemicals and materials

D-Biotin, L-histidine-HCI, NADP, glucose-6-phosphate were purchased from Sigma Chemical Co. (St. Louis, MO). Agar-agar, and other chemicals were purchased from Merck KgaA (Darmstadt, Germany). Dulbecco's modified Eagle's medium (D-MEM) was purchased from Gibco/BRL Products (Grand Island, NY). Fetal bovine serum (FBS) was obtained from HyClone (Road Logan, UT). Culture dishes and flasks were obtained from Costar (Corning, NY).

2.2. Preparation of the saffron extract

Stigmata of pure saffron (Mancha, Spain) were purchased from a local market and stored in the dark at 4 °C. The concentrated saffron extract was prepared as previously described (Abdullaev and Frenkel, 1992a,b). The final concentration of the saffron extract in the cultures ranged from 50 to 1500 μg/plate for *Salmonella typhimurium,* and from 50 to 400 μg/ml for cultured tumor cells, as indicated in the text.

2.3. Cell culture

For our experiments, we chose normal and three types of human malignant cells: CCD-18Lu (human normal lung cells), HeLa (human cervix epitheloid carcinoma), A-204 (human rhabdomyosarcoma) and HepG2 (human hepatocellular carcinoma) cells obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in D-MEM with 10% FBS in a CO_2 water-jacketed incubator (Nuaire, Plymouth, MN) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Incubation with the saffron extract and its ingredients were carried out under the same conditions as previously described (Abdullaev and Frenkel, 1992a,b).

2.4. Colony formation assay

Tested cells (2×10^5) were seeded into 100-mm Petri dishes with 10 ml D-MEM medium. After 2 days, different concentrations of tested agents were added and incubation continued for 3 h. The cells were then trypsinized (0.05 M trypsin), counted and 200 cells were seeded in 60-mm Petri dishes for colony formation estimation. At least three replicate colony determinations were carried out for each culture. After 10 days of incubation, the resulting colonies were rinsed with 50 mM phosphate buffer, pH 7.6, containing 150 mM NaCl, fixed with methanol, stained with Giemsa (Sigma) and the number of colonies (with diameter >0.05 mm) per dish were determined as previously described (Abdullaev and Frenkel, 1992b).

2.5. Antimutagenicity assays

Dr. B.N. Ames, at the University of California in Berkeley kindly provided the *S. typhimurium* TA98 tester strain. The mutagenic and the antimutagenic activities of the saffron extract and its ingredients were determined using the plate incorporation test (Maron and Ames, 1983) and the following mutagens: benzo[a]-pyrene (BP) and 2-amino-antracene (2-AA). BP (10 μg/plate) and 2-AA (20 μg/plate) were chosen as positive controls for the mutagenicity studies, since this dose is not toxic for the TA98 strain. 0.5 ml of S9 mixture (10% rat liver S9 fraction, 8 mM $MgCl_2$, 33 mM KCl, 4 mM NADP, 5 mM glucose-6-phosphate and 100 mM sodium phosphate buffer (PBS), pH 7.4 were added to the molten top agar before plating. The S9 fraction was obtained from Aroclor 1254 treated rats as previously described by Maron and Ames (1983). Toxicity of the tested agents was assessed by the observation of the background bacterial growth in the minimal agar plates due to traces of histidine in the medium (Ames et al., 1975). Revertant colonies were counted after a 72-h incubation period at 37 °C using a MiniCount colony

counter (Biotran II, New Brunswick Scientific, Edison, NJ).

Each plate was examined for signs of possible toxicity, such as thinning of the background lawn, or reduction of spontaneous counts. None of the treatments reported here were toxic to the bacteria. Experiments were repeated at least twice, using triplicate plates for each dose.

The mutation index was calculated as $MI = x_1/x_0$, where x_1 is the revertant colonies at each dose of the agent assayed, and x_0 is number of revertants at negative control. A test compound was considered mutagenic if the number of the His+ revertant colonies was increased at least twice the value of the corresponding control ($MI > 2$), over at least three dose levels, and a reproducible dose–response curve could be demonstrated.

2.6. Statistical analysis

Results are expressed as the mean ±S.E.M. of triplicate plates. Each experiment was repeated at least twice. Data were analyzed using Statistical Analysis System software (SAS Institute Inc., Cary, NC 27511, USA Release 6.02). Values were considered significant when $P < 0.05$.

3. Results

3.1. Cytotoxic effect of the saffron extract on tumor cells

We have examined the effect of different concentrations (from 50 to 400 μg/ml) of saffron extracts on colony formation of normal (CCD-18Lu) and malignant human cells (HeLa, A-204 and HepG2 cells) in vitro. Results of these experiments (Fig. 1) demonstrated that the saffron extract had no effect on the CCD-18Lu cells, but inhibited colony formation of all tested malignant cell lines in a dose-dependent manner. All tested malignant cells showed a good response to the effect of the saffron extract, but the A-204 cell line showed a higher sensitivity to the inhibitory effect of the saffron extract. We also examined the effect of different ingredients of saffron on colony formation of human tumor cells (Table 1). We chose HeLa cells for this experiments, since this cell line is easier to cultivate in comparion to other cell lines, and cervical cancer is major health issue affecting a great number of Mexican women (21%). All carotenoid components of saffron inhibited tumor cell growth. Trans-crocin 3 showed a stronger inhibitory effect in comparison with other saffron components.

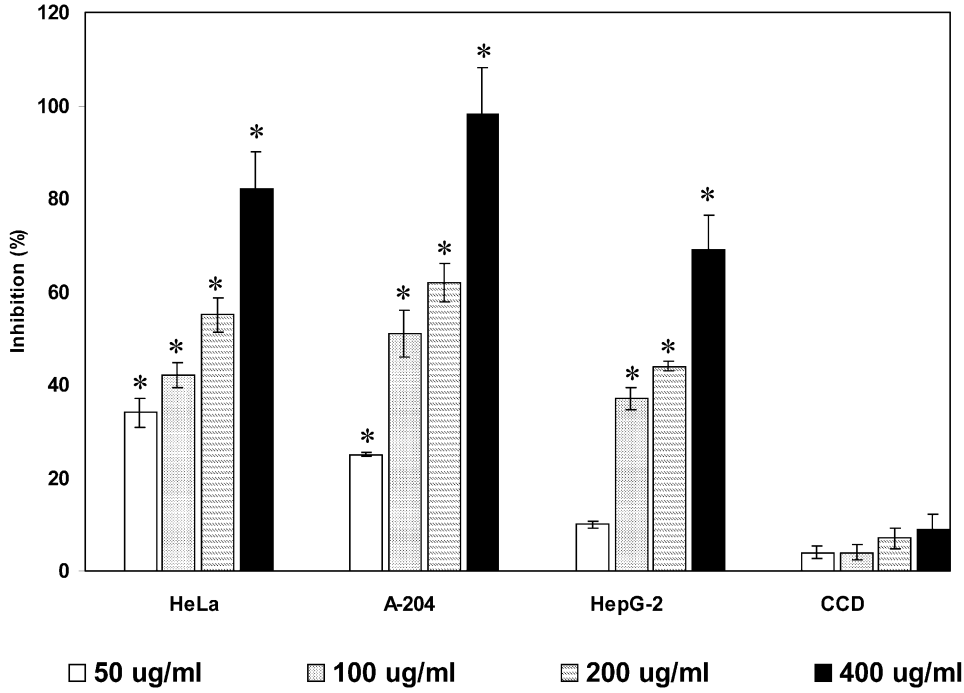

Fig. 1. Inhibitory effect of saffron extract on colony formation of different human cells. Cells were exposed to the indicated concentrations of saffron extract for 3 h. The number of cells that were able to form colonies was determined as described in Materials and methods section. Results are presented as the percentage of the inhibition of colony formation (colonies/dish formed by untreated cells accepted as 100%; for CCD-18Lu = 28; HeLa cells = 68; for A-204 cells = 60; for HepG2 cells = 58). Each value represents the mean ± S.D. of triplicate plates. *$P < 0.05$.

3.2. Antimutagenic activity of the saffron extract

No marked effect of the saffron extract on BP-induced mutagenic activity including the highest concentration used (1500 μg/plate) was seen (Table 2) in the Ames/*Salmonella* test system with the TA98 strain + S_9. Although all tested concentrations of the saffron extract slightly reduced BP mutagenicity, no clear dose–response effect was obtained. The saffron extract induced a comutagenic effect on 2-AA induced mutagenicity in a dose-dependent manner, although saffron itself had no any mutagenic activity (Table 2). The saffron extract at a concentration of 1500 μg/plate more than tripled 2-AA's mutagenic activity. Examination of the effects of four main ingredients of the saffron extract demonstrated that safranal may be one of the components responsible for this co-mutagenic effect (Table 3). After using the plate incorporation assay, no mutagenic activity of the saffron extract up to the concentration of 1500 μg/plate was detected (Table 4) when using the *S. typhimurium* tester strain TA98, both with and without S9 activation. The mutation index (MI) was less than 2 at all tested saffron extract concentrations.

Table 1
Inhibitory effect (%) of different saffron ingredients on colony formation in human tumor HeLa cells

Saffron ingredients (200 μg/ml)	% of inhibition
Crocin 1	7.0±0.9
Crocin 2	10.0±1.2
Crocin 3	25.0±2.1
Picrocrocin	27.0±2.7
Picrocrocin (acid forms)	30.0±1.9
trans-Crocin 2′	10.0±1.6
trans-Crocin 3	36.0±2.4
trans-Crocin 4	27.0±2.3
cis-Crocin 3	18.0±1.8
Crocetin	25.0±2.0

HeLa cells were exposed to indicate concentrations of saffron ingredients for 3 h. The number of cells that were able to form colonies was determined as described in Section 2. Results are presented as the percentage of inhibition of colony formation (colonies/dish) formed by untreated cells 100% (HeLa cells = 70). Each value represents the mean±S.D. of triplicate plates.

Table 2
Comutagenicity of the saffron extract, assayed in the *Salmonella typhimurium* plate incorporation test, with metabolic activation (S_9mix) and 10 μg per plate of BP or 20 μg per plate of 2AA

His^+Rervertants per plate (mean±S.D.)[a]		
TA98		
Test agents (μg/plate)		MI
None	36±2.3	–
Saffron extract + BP		
100	339±31.2	9.4
200	341±33.4	9.4
500	347±30.0	9.6
1000	344±30.4	9.5
1500	343±32.7	9.5
BP (10 μg per plate)	391±19.5	10.8
None	32±3.6	–
Saffron extract + 2AA		
100	1006±95.0	31.4
200	1281±44.2	40.0
500	1667±29.0	53.1
1000	2479±87.5	77.5
1500	2970±97.7	92.8
2AA (20 μg per plate)	915±113	28.6

[a] Each value represents the mean±standard deviation (S.D.) of triplicate plates MI: Mutation Index.

4. Discussion

Chemoprevention involves the use of naturally occurring and synthetic agents (alone and in combination) to prevent cancer. This is an important facet of biomedical

Table 3
Comutagenicity of some ingredients of saffron, assayed in the *Salmonella typhimurium* plate incorporation test, with metabolic activation (S_9mix) and 20 μg per plate of 2AA

His^+Rervertants per plate (mean±S.D.)[a]		
TA98		
Test agents (nM/plate)		MI
Crocin		
None	39±12.2	–
100	403±37.6	10.1
200	359±32.5	9.2
400	348±54.3	8.9
2AA (20 μg per plate)	440±40.8	11.3
Kaempferol		
None	43±10.1	–
100	286±73.2	6.6
200	276±43.1	6.4
400	307±20.8	7.1
2AA (20 μg per plate)	319±7.8	7.4
Picrocrocin		
None	27±3.6	–
100	85±13.1	3.1
200	103±14.4	3.8
400	84±10.1	3.1
2AA (20 μg per plate)	103±10.1	3.1
Safranal		
None	27±4.4	–
100	560±10.0	20.7
200	685±31.9	25.4
400	766±46.5	28.4
2AA (20 μg per plate)	304±17.5	11.3

MI: Mutation Index.
[a] Each value represents the mean±standard deviation (S.D.) of triplicate plates.

Table 4
Non-mutagenic activity of saffron extract assayed in the *Salmonella typhimurium* TA98 strain plate incorporation test, with and without metabolic activation (S_9mix)

His^+ Revertants per plate (mean±S.D.)[a]			
TA98			
Saffron extract (μg/plate)	x±S.D. without S9 mix	x±S.D. with S9 mix	MI
None	39±9.5	45±6.7	–
50	33±7.1	41±8.3	0.9
100	28±10.3	35±5.4	0.8
200	26±12.7	37±7.3	0.8
300	25±9.2	35±8.9	0.8
500	24±8.1	36±7.1	0.8
1000	30±3.9	38±6.5	0.8
1500	22±5.0	34±3.8	0.7
BP (10 μg per plate)	24±6.8	391±19.5	8.7
2AA (20 μg per plate)	32±3.6	915±9.7	20.3

MI: Mutation Index.
[a] Each value represents the mean±standard deviation (S.D.) of triplicate plates.

research, providing a practical approach for identifying potentially useful inhibitors of tumor development, and offering an opportunity to study the molecular mechanism of tumorigenesis.

It is known that mutations are important early steps in carcinogenesis, therefore, short-term genetic test such as the *Salmonella*/reversion assay, has been successfully used for the detection of mutagens/carcinogens, as well as of antimutagens/anticarcinogens (Rausher et al., 1998).

It was reported that the oral LD_{50} for saffron in mice was 20.7 g/kg administered as a decoction. Higher doses can be fatal, since saffron contains a poison that acts on the central nervous system and damages the kidney (Chang et al., 1964). Our study showed that the oral administration of the saffron extract at concentrations from 0.1 to 5 g/kg was non-toxic in mice (Abdullaev et al., 2002).

A number of previous studies suggest that saffron possesses antitumor and anticancerogenic activities and has no cytotoxic effect on non-malignant cells (Salomi et al., 1991; Nair et al., 1991a,b, 1994, 1995; Abdullaev and Frankel, 1992a,b; el Daly, 1998; Escribano et al., 2000). It is interesting to note that saffron exhibited cytotoxic inhibitory activity against different animal and human malignant cells (Abdullaev, 1993, 2002; Nair et al., 1995; Ríos et al., 1996; Abdullaev and Frenkel, 1999; Winterhalter and Straubringr, 2000).

Our results indicate that the saffron extract (Fig. 1) had a dose-dependent inhibitory effect on colony formation of all tested tumor cells in vitro, but had no effect on normal human cells. Potential compounds responsible for saffron's inhibitory effect on tumor cell growth are its carotenoid ingredients (Table 1).

With respect to the mechanism(s) that may be involved, the intracellular level of sulphydril (SH) compounds in tumor cells may be important factors partaking in the relative sensitivity of malignant cells to the effect of saffron (Abdullaev and González de Mejía, 1995). We previously demonstrated that the pre-treatment of tumor cells with saffron resulted in a doubling of intracellular SH-compound levels (Abdullaev and González de Mejía, 1995). It seems that the marked difference in the inhibition of colony formation in tumor cells by saffron may be explained by an increase in the levels of intracellular SH-compounds in tested malignant cells (Abdullaev and Frenkel, 1992a,b, 1994; Abdullaev and González de Mejía, 1995). It is evident from our results that the saffron extract exhibited non-mutagenic (Table 4) and non-antimutagenic (Table 2) effects on BP mutagenicity in the *Salmonella* tester strain TA98. We also observed that saffron increased mutagenicity (Table 2) of 2-AA and this co-mutagenic effect depended on the concentration of saffron. Finally, it was demonstrated that safranal was potentially responsible for the co-mutagenic effects of saffron on 2-AA mutagenicity (Table 3).

The reason for the variation in the number of mutant colonies observed in Table 3, could be due to the different time in which experiments with each of saffron compounds were done. The distribution of mutagenic signals in response to a single dose of mutagen requiring or not metabolic activation is unusually broad, presumably reflecting not only the genetic variability of the microorganism but also the diferences in activity of the various batches of S9 preparations (Rosenkranz et al., 1983).

Additional studies are required for determining the biological effect of the saffron ingredients, especially concerning the co-mutagenic effect of saffron on 2-AA mutagenicity.

Thus, these results reveal that the saffron extract is non-toxic, non-comutagenic with BP, but exhibits a co-mutagenic effect with 2-AA. Saffron possessed cytotoxic activity against different tumor cells, while no inhibitory effect is exerted on normal in vivo cell growth.

In conclusion, the present study supports increasing evidence that naturally occurring saffron extract may have an important role in cancer chemoprevention.

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