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Protective effect of saffron (*Crocus sativus* L.) aqueous extract against genetic damage induced by anti-tumor agents in mice

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The genotoxic potential of anti-tumor drugs limits their efficacy in the treatment of cancers. Since ancient times, saffron (dried stigmas of *Crocus sativus* L.) has been used as a spice and medicinal herb. Saffron is a rich source of carotenoids and is known for its anti-cancer and anti-tumor properties. The present study was designed to ascertain the chemoprotective potential of saffron against the genotoxicity of three well-known anti-tumor drugs – cisplatin (CIS), cyclophosphamide (CPH) and mitomycin-C (MMC) – using comet assay. Three doses of saffron (20, 40 and 80 mg/kg b.w.) were orally administered to mice for five consecutive days prior to the administration of anti-tumor drugs under investigation. Pre-treatment with saffron significantly inhibited anti-tumor drugs induced cellular DNA damage (strand breaks) as revealed by decreased comet tail length, tail moment and percent DNA in the tail. These findings, together with our previous results, suggest a potential role for saffron as an anti-genotoxic, anti-oxidant and chemopreventive agent and could be used as an adjuvant in chemotherapeutic applications. *Human & Experimental Toxicology* (2006) **25,** *79–84*

Key words: anti-tumor; cisplatin; comet assay; cyclophosphamide; genotoxicity; mitomycin-C; saffron

Introduction

There has been increasing awareness of the genotoxicity of anti-cancer drugs due to their serious side-effects and possible induction of secondary malignancies. Epidemiological surveys and laboratory investigations have yielded substantial evidence to establish that dietary agents can play an important role in inhibiting genotoxicity and carcinogenicity.[1–5] Dietary intake of such chemopreventive/chemoprotective agents has been regarded as an effective strategy in preventing the deleterious effects of genotoxins and/or carcinogens.[6–8] However, their effective use requires assessment of their potential and an understanding of their probable mechanisms of action.

Saffron, obtained from dried stigmas of *Crocus sativus* L. (Iridaceae), is a highly valued spice, commonly used in flavoring and coloring food in different parts of the world and is known to possess the richest source of carotenoids.[9–11] It has been used in traditional medicine as an aphrodisiac, antispasmodic and expectorant, nerve sedative and stomachic.[12] During the last few years, the antitumoral properties of saffron have been demonstrated. It was shown that saffron extract and its characteristic components possess anti-carcinogenic and anti-tumor activities *in vivo* and *in vitro.*[13–18] Previously, we observed that pre-treatment with saffron decreased the frequency of bone marrow micronuclei and the extent of hepatic oxidative stress in mice treated with various genotoxins, viz. cisplatin (CIS), cyclophosphamide (CPH) and mitomycin-C (MMC) and urethane.[19,20]

In view of the above findings, the present study was designed to further evaluate the potential chemoprotective effects of orally administered saf-

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fron on the genotoxicity of three well-known anti-tumor drugs – CIS, CPH and MMC – with different mechanisms of action,[21–23] employing comet assay. Single-cell gel electrophoresis (or comet assay) is a rapid and sensitive fluorescence microscopic method for the detection of DNA damage (specifically single strand breaks and alkaline-labile sites) at individual cell level.[24,25]

Materials and methods

Animals and diet

All experiments were carried out on 10- to 12-week-old male Swiss albino mice, weighing 25–30 g. These animals were obtained from the National Institute of Nutrition (NIN), Hyderabad, India and maintained in the University Animal House on the standard mouse diet (pellets from Hindustan Lever Ltd, Mumbai, India) and water *ad lib*. The animals used in the present study were maintained in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and approved by the Institute's ethical committee.

Chemicals

Saffron (dried stigmas of *C sativus* L.) was purchased from the Indian Medical Practitioners Co-Operative Pharmacy and Stores (IMPCOPS), Chennai, India. CPH and MMC were purchased from Sigma Chemical Company (St. Louis, USA). URE and CIS were obtained from Fluka (Switzerland) and Tamil Nadu Dhada Pharmaceuticals Ltd. (Chennai, India), respectively.

Preparation of aqueous extract of saffron

Saffron (1 g) was soaked in distilled water (100 mL). After 2 hours, it was homogenized in the same distilled water, stirred for 1 hour and filtered. The residue was re-extracted with fresh distilled water. This aqueous extract was lyophilized and stored at 4°C until further use.

Pre-treatment with saffron and exposure to the anti-tumor drugs

Ninety-six mice were divided into four major groups (Groups I–IV). Each group was further subdivided into four groups: A, B, C and D, of six animals each. Animals in subgroups B, C and D, were given by gavage (10 mL/kg b.w.) 20, 40 and 80 mg/kg b.w. of freshly prepared saffron extract, respectively, for five consecutive days. Duration of pre-treatment and test doses of saffron were decided on the basis of findings from our previous studies.[19,20] Two hours after administration of the final dose of saffron extract, the anti-tumor drugs, CIS (5 mg/kg b.w.), CPH (40 mg/kg b.w.) and MMC (1 mg/kg b.w.), were dissolved in saline and injected intraperitoneally (10 mL/kg) into the experimental Groups II, III, and IV, respectively (except Group I). Control animals (Group Ia) received the same volume of distilled water. All the experimental animals were sacrificed by cervical decapitation 24 hours after the treatment. Both femurs were removed immediately and processed as follows.

Extraction of femoral bone marrow

Cells from bone marrow were obtained according to the method described by Tice.[25] The bone marrow cells were extracted from the femur into Hanks Balanced Salt Solution (HBSS) (2 mL) containing 20 mM disodium ethylenediaminetetraacetic acid (EDTA) using a 21-gauge needle. The femur was cleaned using a piece of gauze before the ends of the bone were excised past the opening to the bone marrow canal. The needle was then inserted into the bone and a flushing solution was passed through to remove the bone marrow.

Single-cell gel electrophoresis assay

Alkaline single-cell gel electrophoresis was performed according to the method of Singh *et al*.,[24] with minor modifications. The bone marrow cells were checked for viability using Trypan blue exclusion technique. Cells isolated from the bone marrow were resuspended in HBSS at a concentration of $(2–5) \times 10^4$ cells/mL and 5 μL of this suspension was mixed in 95 μL of 0.75% low melting point agarose. The mixture (cell/agarose) was added to a fully frosted microscopic slide coated with a layer of 300 μL of normal melting agarose (1%). After solidification, the slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0–10.5, with freshly added 1% Triton X-100 and 10% DMSO) for 24 hours (in darkness). Subsequently, the slides were incubated in fresh alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 12.6) for 30 min. The DNA was electrophoresed for 30 min at 300 mA and 25 V (0.90 V/cm). The slides were neutralized with 0.4 M Tris (pH 7.5), followed by rinsing with distilled water and methanol, and were then stained with ethidium bromide (2 μg/mL).

All the slides were scored using Nebug, an image analysis system attached to a fluorescence microscope equipped with appropriate filters. The microscope was connected to a computer through a charge coupled device (CCD) camera to transport images to software for analysis. The final magnification was ×400, the parameters taken were: tail length (migration of the DNA away from the nucleus (μm)),

tail moment (arbitrary units) and tail DNA (%). Seventy-five individual cells (25 cells from each of three replicated slides) were analysed per sample.

Statistical analysis

Treatment effects were analysed by one-way ANOVA using GraphPad Prism version 4.0 (GraphPad Software). When a *P*-value obtained from ANOVA was significant, Tukey's test was applied to test for differences among groups. Differences were considered significant if $P < 0.05$. Values in Table 1 are the mean ± SD for six mice in each group.

Results

Cell viability assessed using Trypan blue was in the range of 90–95%. The results of the comet assay, namely tail length (TL), tail moment (TM) and percent DNA in tail (TDNA), in the bone marrow cells of mice dosed with saffron at 20, 40 and 80 mg/kg b.w. besides negative and positive controls (CIS, CPH and MMC) are presented in Table 1. The observed TL, TM and TDNA in the anti-tumor drugs alone treated animals were significantly higher ($P < 0.001$) than the values of the negative control. All three doses of saffron were effective in exerting significant ($P < 0.001$) protection against the genotoxicity of anti-tumor drugs under investigation, as evidenced from the reduction in the observed TL, TM and TDNA compared to their respective positive control (genotoxins alone treated group). The maximum reduction in the TL (40%), TM (47%) and TDNA (52%) was observed in mice pre-treated with 40 mg/kg saffron. Furthermore, mice treated with the highest test dose of saffron alone did not show any statistically significant DNA damage.

Frequency distribution histograms of TM with respect to percent cells from various treated groups, such as control (Figure 1a), saffron and CIS (Figure 1b), saffron and CPH (Figure 1c) and saffron and MMC (Figure 1d) are depicted in Figure 1. In the case of saffron control, around 80% of cells showed 0–2.5 μM TM and <2% of cells showed >10 μM TM (Figure 1a). Whereas in anti-tumor drugs alone treated mice, around 15–20% of cells showed 0–2.5 μM TM and >15% cells had >10 μM TM. However, pre-treatment with different doses of saffron decreased the percentage of cells with higher TM compared to genotoxin alone treated population.

Discussion

Recently, considerable attention has been focused on the use of dietary constituents as chemopreventive/chemoprotective agents for the control of cancer and other related genetic diseases. Cytogenetic biomarkers have assumed significance as reliable, early indicators of biological effects of genotoxin/carcinogen induced DNA damage owing to the strong association between specific chromosomal alterations and tumorigenesis.[26] Bone marrow cytogenetics is a useful short-term technique for elucidating the mechanism as well as identifying the substances for their clastogenic and anti-clastogenic activity.[27] We, therefore, evaluated the effects of

Table 1 Tail DNA, tail length and tail moment in control and experimental groups

Group (genotoxin)	*Sub group*	*Saffron (mg/kg b.w.)*	*Tail DNA (%) mean ± SD*	*Tail length (μm) mean ± SD*	*Tail moment mean ± SD*
Group I (control)	A	0	8.31 ± 1.76	1.05 ± 0.14	0.80 ± 0.07
	B	20	7.34 ± 0.89	1.04 ± 0.08	0.76 ± 0.06
	C	40	7.84 ± 0.72	0.99 ± 0.11	0.83 ± 0.05
	D	80	8.72 ± 1.04	0.98 ± 0.11	0.86 ± 0.06
Group II (CIS) 5 mg/kg b.w.	A	0	26.73 ± 3.43*	3.00 ± 0.26*	7.46 ± 0.68*
	B	20	20.08 ± 2.14*	2.27 ± 0.36*	4.86 ± 0.51*
	C	40	15.84 ± 1.86*	1.59 ± 0.21*	3.51 ± 0.39*
	D	80	18.57 ± 2.17*	1.85 ± 0.37*	4.22 ± 0.44*
Group III (CPH) 40 mg/kg b.w.	A	0	18.43 ± 2.06*	1.68 ± 0.22*	4.61 ± 0.49*
	B	20	13.69 ± 1.40*	1.21 ± 0.16*	2.21 ± 0.18*
	C	40	11.23 ± 1.17*	1.10 ± 0.12*	1.58 ± 0.21*
	D	80	12.89 ± 1.20*	1.16 ± 0.13*	2.08 ± 0.26*
Group IV (MMC) 1 mg/kg b.w.	A	0	21.08 ± 1.94*	2.29 ± 0.27*	5.36 ± 0.60*
	B	20	17.61 ± 1.83**	1.75 ± 0.27**	3.32 ± 0.39*
	C	40	14.32 ± 1.30[j]*	1.26 ± 0.20*	2.63 ± 0.23*
	D	80	15.76 ± 1.71[i]*	1.45 ± 0.38*	3.16 ± 0.36*

Data showing chemoprotective effects on mice treated with different doses of saffron. Groups were treated as mentioned in materials and methods section. Comparisons were made between Group IA and Groups IIA, IIIA, IVA; subgroup A compared with subgroup B, C and D, in respective groups.
*Significantly different from the respective controls at $P < 0.001$ (Tukey's test).
**Significantly different from the respective controls at $P < 0.05$ (Tukey's test).

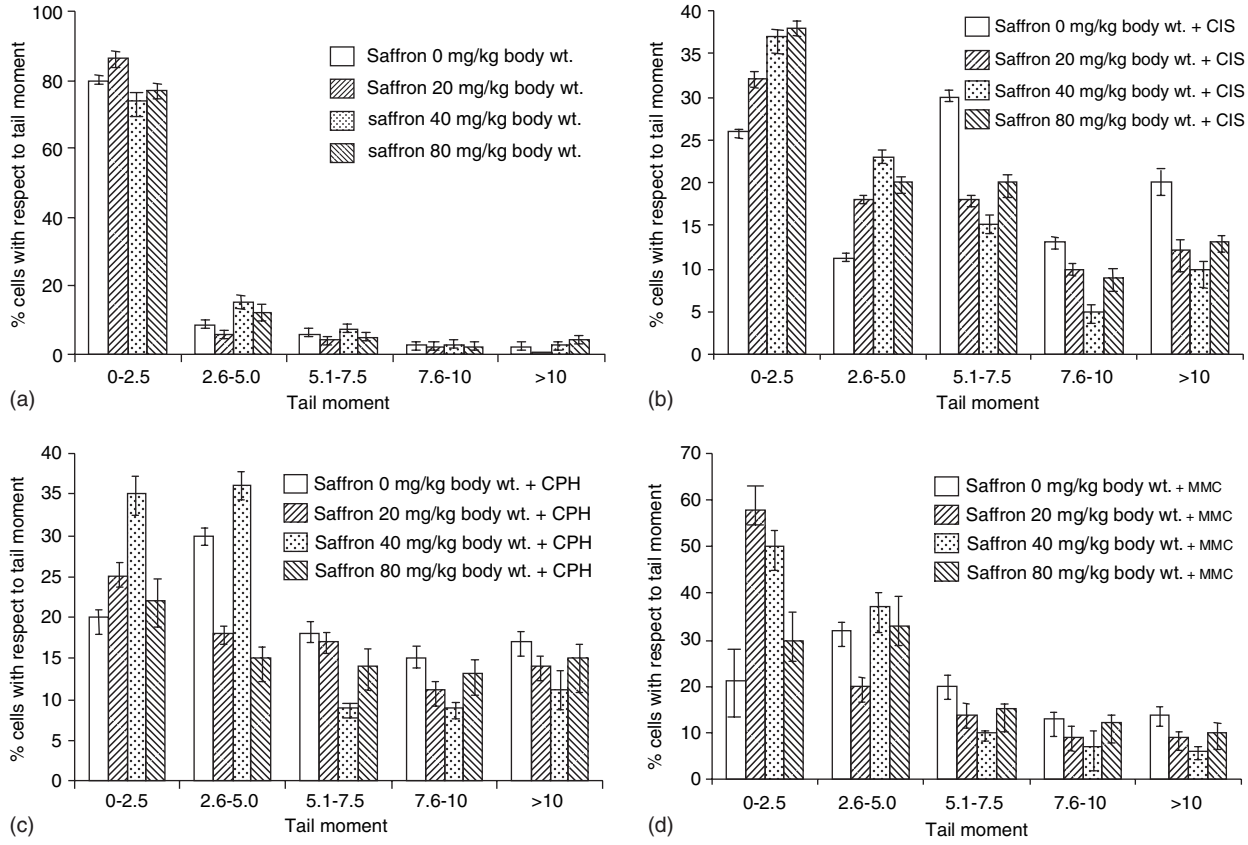

Figure 1 (a) Percentage distribution of cells with respect to tail moment after treatment with different doses (0, 20, 40 and 80 mg/kg b.w. for five consecutive days) of saffron alone. (b) Percentage distribution of cells with respect to tail moment after treatment with different doses of saffron (0, 20, 40 and 80 mg/kg b.w for five consecutive days) on cisplatin (5 mg/kg b.w.) induced DNA damage. (c) Percentage distribution of cells with respect to tail moment after treatment with different doses of saffron (0, 20, 40 and 80 mg/kg b.w. for five consecutive days) on cyclophosphamide (40 mg/kg b.w.) induced DNA damage. (d) Percentage distribution of cells with respect to tail moment after treatment with different doses of saffron (0, 20, 40 and 80 mg/kg b.w. for 5 consecutive days) on mitomycin-C (1 mg/kg b.w.) induced DNA damage.

saffron on chemically induced genetic damage in mouse bone marrow by quantification of DNA damage using comet assay as an end-point.

The results of the present study revealed the chemoprotective potential of saffron. Our findings show that saffron can play a role in the process of reducing the *in vivo* genotoxicity of anti-tumor agents. The fact that the protection against the DNA strand breaks induced by different anti-tumor drugs used in this investigation shows that saffron may also be used as an adjuvant in chemotherapy. Pre-treatment was carried out with different doses of aqueous extract of saffron, to understand the possible dose dependent effects. However, the observed anti-genotoxic effects were not always dose-dependent (Table 1). The maximum inhibition of genotoxicity was observed after pre-treatment with a dose of 40 mg/kg b.w. A further increase in dose did not enhance this inhibitory effect. Furthermore, there is no indication of any significant increase in genotoxicity after the 5-day pre-treatment with 20, 40 or 80 mg/kg b.w. of saffron alone.

Despite the obvious differences in the mechanisms of action and metabolic activation of the anti-tumor drugs employed in this investigation, overall findings show a clear reduction ($P < 0.001$) of DNA damage in terms of tail length, tail moment and percent tail DNA in the bone marrow cells of animals pre-treated with different doses of saffron. CIS is a directly acting alkylating agent, which, upon hydrolysis in aqueous solution, forms various reactive species responsible for DNA damage.[28] CPH requires metabolic activation by the cytochrome P-450 dependent mono-oxygenase system, damages chromosomes through the generation of free radicals and alkylating DNA, thereby producing mutagenicity.[22] MMC is activated by a widely distributed NADPH-dependent quinine reductase to mono-functional and bi-functional alkylating derivatives. DNA damage occurs indirectly via reactions involving reactive oxygen species generated during reductive activation of mitomycin C.[29]

The protective effects of saffron observed in the present study may be related to the anti-oxidant

properties of their constituents, such as crocin, crocetin and safranal. Although the present study did not aim to identify the main active ingredients responsible for the protective action, some authors have assessed fractions and extracts to determine the anti-oxidant and anti-tumor properties of saffron. In rats, crocin dyes are known to exert protective effects against acute hepatic damage induced by aflatoxin B_1 and dimethylnitrosamine. It has been shown that crocetin, the deglycosylated crocin derivative, has protective effects on aflatoxin B_1-induced hepatotoxicity and protects rat primary hepatocytes against oxidative damage.[30,31] Cancer chemopreventive, as well as anti-tumor activities, were also reported for crocins and crocetin derivatives in different assay systems.[16–18,30] It is possible that some of these compounds possess the anti-mutagenic activity. Chemopreventive agents can exert their anti-mutagenic/anti-carcinogenic effects by inhibiting the formation of reactive mutagenic/carcinogenic metabolites, induction of enzymes that detoxify carcinogens, scavenging reactive oxygen species, influencing apoptosis and inhibiting cell proliferation.[32,33]

Many chemopreventive compounds are known for their ability to induce a set of enzymes involved in genotoxic/carcinogenic detoxification.[34–37] Our earlier studies indicated a saffron mediated increase of glutathione (GSH)-related enzymes in genotoxin treated animals.[21] Nair *et al*. observed that saffron increased the intracellular levels of reduced glutathione and glutathione related enzymes and suggested possible anti-oxidant activity of saffron.[38] It has been speculated that saffron induced the elevation of reduced-GSH and related enzymes probably due to the presence of carotenoids, which might act as either membrane associated high efficiency free radical scavengers or serving to maintain the functional levels of other closely related anti-oxidants in a cell pool. Glutathione *S*-transferase (GST) catalyses the conjugation of GSH with a variety of reactive electrophiles and it takes on considerable importance as a mechanism for carcinogen detoxification and cellular protection. Agents inducing GST or increasing the pool of GSH in the cells are generally known to inhibit genotoxicity and carcinogenicity.[39,40] Our previous findings have shown that saffron attenuated the inhibition of GST activity by these anti-tumor drugs.[20,21] Hence, the anti-oxidant activity of saffron seems to be a likely explanation for the observed inhibition of genotoxicity. Nevertheless, since the metabolic activation of CPH is known to involve cytochrome P-450 activities, one cannot rule out the possibility of saffron exerting its protective effect by inhibiting the activation of process, in addition to other mechanisms, such as complex formation. The findings of our present study and some recent reports have shown that saffron extract and some of its constituents can inhibit the genotoxicity/carcinogenicity of chemicals with various mechanisms of action. These observations indicate that more than one mechanism of protection is operating.

This result gives a first indication of the anti-mutagenic action of saffron extract against different DNA damaging agents. It complements our earlier studies showing anti-clastogenic activity of saffron extract in the mouse bone marrow.[20] In conclusion, the findings from this present study indicate that saffron may be regarded as a valuable plant source for use in traditional medicine and modern drug development. However, further studies are required to elucidate the mechanism of *in vivo* chemoprotection by saffron against diverse DNA damaging agents.

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