RESEARCH COMMUNICATION

Saffron Can Prevent Chemically Induced Skin Carcinogenesis in Swiss Albino Mice

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Abstract

One of the most promising strategies for cancer prevention today is chemoprevention using readily available natural substances from vegetables, fruits, herbs and spices. Among the spices, saffron (Crocus sativus, L) a member of the large family Iridaceae, has drawn attention because apart from its use as a flavouring agent, pharmacological studies have demonstrated many health promoting properties including radical scavenging, anti-mutagenic and immuno-modulating effects. In the present study the effects of an aqueous infusion of saffron on two stage skin papillogenesis / carcinogenesis in mice initiated by 7-12 dimethyl benz[a] anthracin (DMBA) and promoted with croton oil were investigated. Significant reduction in papilloma formation was found with saffron application in the pre-initiation and post-initiation periods, and particular when the agent was given both pre- and post-initiation. The inhibition appeared to be at least partly due on modulatory effects of saffron on some phase II detoxifying enzymes like glutathione-S-transferase (GST) and glutahione peroxidase (GPx), as well as catalase (CAT) and superoxide dismutase (SOD).

Key Words: Saffron - skin carcinogenesis - detoxification enzymes - chemoprevention

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Introduction

Saffron, the dry stigma of the plant Crocus sativus L popularly used as a spice and food colorant, has been used in traditional medicines for treatment of many diseases including tumors. The chemical components of saffron include the coloured carotenoids, crocin and crocetin, and the monoterpenic aldehydes, picrocrocin and safranal (Abdullaev 1993, Tarantilis et al, 1995; Escribano et al, 1996, Lozano et al, 2000). In addition saffron contains proteins, sugars, vitamins, flavonoids, amino-acid, minerals and gums (Abdullaev 1993, Rios et al 1996). A number of reports have indicated anti-tumor properties of saffron extract in vivo (Salomi et al 1991, Abdullaev et al 1992, Abdullaev et al 2002). Anti-promoting and non-mutagenic activity of saffron extract has also been reported (Salomi et al 1991). It has also been shown that crocins can protect from the adverse effects of hepatocarcinogenic compounds (Lin and Wang 1986) and that crocetin, the deglycosylated crocin derivative, can inhibit intracellular nucleic acid synthesis (Abdullaev 1994). In vitro proliferation of promyelocytic leukemia cells is inhibited by crocins and crocin derivatives (Tarantitis et al, 1994). Another study (el Daly 1998) demonstrated protective effects of saffron extract together with cysteine and vitamin E against cisplatin induced toxicity in rats. However, to our knowledge there has been no systematic cytotoxicity analysis of saffron compounds, crocin, crocetin, picrocrocin, and safranol performed separately. In the present study we examined the anti carcinogenic effects of aqueous infusion of saffron, administered orally using two stage skin carcinogenesis model in mice. Since phase II detoxification enzymes play a major role in the onset of carcinogenesis by helping eliminating the damaging agents, modulatory effects of saffron treatment on a number of enzyme species in the same model were also ascertained.

Materials and Methods

Preparation of Test Material

Commercially available saffron was purchased from the Govt. of Kashmir Emporium, Kolkata and stored at 4°C until use. An infusion was prepared by soaking 0.1 gm saffron in 12.5ml of lukewarm distilled water for 10 minutes followed by straining. The infusion was administered orally at a dose of 200 mg/kg body weight /mouse.

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Preparation of Skin for Histology

Each set consisted of 12 mice. The affected skin was collected for histology.

Morphological Observation of Papilloma Development

2 weeks of application of DMBA/croton oil.

Saffron infusion at different doses (50-500 mg/kg body weight by oral gavage) were administered to normal mice. Based on body weight, skin texture, food intake and general behavior observed for 7 days, the dose selected was 200 mg/kg body weight/day/mouse (maximum dose not producing change in any of these parameters).

Chemicals
DMBA (7-12 dimethylbenz [a] anthracene), croton oil, CDNB (1-chloro-2-4-dinitrobenzene), GSH (glutathione), GR (glutathione reductase), NADPH (nicotinamide adenine dinucleotide phosphate reduced form), Pyrogallol, TBA (thiobarbituric acid) and DTPA (diethylene triamine penta acetic acid) and pyridine, were purchased from Sigma Chemicals Co, St. Louis, MO, U.S.A. Sodium dodecyl sulphate (SDS) was purchased from Gibco BRL, U.S.A. and hydrogen peroxide solution (H2O2) (30%), acetic acid (CH3COOH), n-butanol and potassium chloride (KCL) from Merck (India).

Experimental Animals
Female Swiss albino mice aged 5-6 weeks, and weighing 20-22 gm at the commencement, were housed in plastic cages with stainless steel wire lids (6 mice per cage). The housing facility was maintained at 30°C. The animals received standard food pellets (Lipton India Ltd.) and water ad-libitum. To induce carcinogenesis the mice received three applications of 100 nmol DMBA in 100µl acetone at an interval of 2 days on the shaved skin (initiator) followed by 100µl 1 gm% croton oil in acetone (promoter) after a week at the same site twice weekly for 8 weeks.

Treatment Groups
The normal control group (NC) received only distilled water every day during the treatment period, while the carcinogen control group (CC) was given three topical applications of DMBA and then croton oil followed by distilled water. The animals of Group–A (pre treatment) were administered saffron infusion orally once daily for 2 weeks prior to the application of DMBA-croton oil. Group-B (pre+post treatment group) was administration the saffron infusion starting 2 weeks prior to the application of DMBA-croton oil and continued until the sacrifice. Group-C (post treatment) was administered saffron infusions starting after 2 weeks of application of DMBA/croton oil.

Morphological Observation of Papilloma Development
Body weights and papilloma counts in mice of groups NC, CC, A, B, and C were recorded at an interval of 7 days till sacrifice. The first set of mice (Set-I) were sacrificed after 2 weeks and the second set (Set-II) after 12 weeks. Each set consisted of 12 mice. The affected skin was collected for histology.

Preparation of Skin for Histology
Affected skin and skin with papilloma were fixed in 10% buffered formalin and dehydrated with graded alcohols starting from 50% ethyl alcohol to absolute alcohol. Then the tissues were embedded in paraffin after clearing in xylene. Serial microtome sections (4µ) were stained with haematoxylin and eosin (Kehar and Wahi, 1967).

Methods for Biochemical Assays
Liver tissue was sampled for biochemical assays after 2 weeks and 12 weeks from all groups for the estimation of lipid peroxidation (LPO) and to measure the activity of phase II detoxification and other enzymes - glutathione-S-transferase (GST), glutathione peroxidases (GPx), catalase (CAT) and superoxide dismutase (SOD).

GST activity was assayed by the method of Habig et al (1974) following the increase in absorbance at 340nm using 1-chloro-2,4-di-nitrobenzene (CDNB) as the substrate. Specific activity of the enzyme was expressed as formation of 1-chloro-2,4-dinitrobenzene-glutathione (CDNB)-GSH conjugate per minute per mg of protein.

GPx activity was measured by the method of Paglia et al. (1967 ) in the post mitochondrial fraction. The reaction mixture contained NADPH and glutathione reductase. The decrease in absorbance following addition of H2O2 was recorded at 340 nm. Enzyme activity was expressed as nmoles of NADPH utilized/minute/mg protein using molar extinction co-efficient of 1.56x10^5 M^-1 cm^-1.

CAT activity in liver was assayed by the method of Luck (1963). The enzyme activity was determined spectrophotometrically at 250 nm and results were expressed as units/mg protein where the unit was the amount of enzyme that liberates half the peroxide oxygen from H2O2 in 100 seconds at 25°C.

SOD activity was assayed by the method of Marklund et al. (1974). Partial extraction and purification of SOD was performed as described by McCord et al. (1969 ). SOD was determined by quantification of pyrogallol auto oxidation inhibition and the results expressed as units/mg protein. One unit of enzyme activity was defined as the amount of enzyme necessary for inhibiting the reaction by 50%. Auto oxidation of pyrogallol in Tris-HCL buffer (50mM,ph-7.5) was measured by increase in absorbance at 420 nm.

Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as a standard.

Statistical Analysis
Data from different experimental groups were analysed and expressed as mean ± SD. The difference in data from different groups were statistically analysed by Student’s t test. A value of p < 0.05 was considered significant for all experiments.
Results

Effects of Aqueous Infusion of Saffron on Body Weight
Gradual increase in body weights was noted in all animals up to 8 weeks except in those from the CC group. At 12 weeks a sharp difference in body weight was noted between the NC and CC groups. Near normal values were noted in the treatment groups A and B (Figure-1).

Effects of Aqueous Infusion of Saffron on Development of Papillomas
Papillomas started to appear on the region of skin receiving topical application of DMBA and croton oil from the 5th to 6th weeks. Percentages of mice with papilloma continued to increase until 12th week in the CC group. Observation made after 12 weeks of treatment revealed that incidence of papilloma in the carcinogen control group was 90%. Reduction in incidence of papilloma was noted in all treatment groups. (Figure-2).

In Set-I animals which were sacrificed after two weeks of DMBA application no papillomas were visible. In Set II animals appearance of papillomas was delayed from the 5th to the 7th week in Gr A and Gr C and to 9th week in Gr B. Treatment with saffron delayed the onset of papillomas as well as the reduced incidence as compared to group CC. The most potent effect of treatment was noted in Gr B (pre + post treatment group).

Effect of Aqueous Infusion of Saffron on LPO
Lipid peroxidation in liver, expressed as TBARS produced, was found to be reduced from that seen in the CC group following treatment. It can be noted from Fig.-3 and Fig.-4 that the best effect of saffron in preventing carcinogen-induced lipid peroxidation was produced in Gr B where saffron treatment started before application of DMBA-croton oil and was continued for up to week 12. After treatment for 2 weeks, a significant inhibition in lipid peroxidation was noted as revealed by decrease in LPO by 1.42 fold (P<0.001), 1.15 fold (P<0.001) and 1.11 fold (P<0.01) in Gr B, Gr A and Gr C respectively. A similar trend was also noted after 12 weeks of treatment. Considering all treatment groups the most significant effect was found in Group B.

Effect of aqueous infusion of saffron on GST
Activity of GST in mouse liver was found to be lower in CC than NC. The enzyme activity significantly increased in Groups A and B by 1.16 fold (P<0.01) and 1.51 fold (P<0.001), respectively after 2 weeks. After 12 weeks the figures were 1.30 fold (P<0.05) and 1.52 fold (P<0.001). The results of treatment in Group C were not significant in either case (Figs 5 & 6).

Effect of aqueous infusion of saffron on GPx activity
Like GST, GPx was also found to be lower in the CC group in comparison to the NC group. Fig 7 & Fig 8 show significant increase in the activity of this enzyme in Groups

Figure 1. Effects of Saffron Infusion on Body Weights

Figure 2. Effect of Aqueous Infusion of Saffron on Incidence of Skin Papillomas at Different Time Points

Figure 3. Effects of Infusion of Aqueous Saffron on LPO in Mouse Liver after Treatment for 2 Weeks. Data are mean ±SD

Figure 4. Effects of Infusion of Aqueous Saffron on LPO in Mouse Liver after Treatment for 12 Weeks. Data are mean ±SD
Figure 5. Effect of Aqueous Infusion of Saffron on the Activity of GST in Mouse Liver. Data are mean ±SD.

Figure 6. Effect of Aqueous Infusion of Saffron on the Activity of GPx in Mouse Liver. Data are mean ±SD.

Figure 7. Effect of Aqueous Infusion of Saffron in Mouse Liver GPx Activity. Data are mean ±SD.

Figure 8. Effect of Aqueous Infusion of Saffron on GPx Activity in Mouse Liver. Data are mean ±SD.

Figure 9. Effect of Aqueous Infusion of Saffron on the Liver Activity of Catalase. Data are mean ±SD.

Figure 10. Effect of Aqueous Infusion of Saffron on the Liver Activity of Catalase. Data are mean ±SD.

Figure 11. Effect of Aqueous Infusion of Saffron on the Activity of SOD in Mouse Liver. Data are mean ±SD.

Figure 12. Effect of Aqueous Infusion of Saffron on the Activity of SOD in Mouse Liver. Data are mean ±SD.
A and B by 1.10 fold (P<0.1) and 1.16 fold (P<0.01) after 2 weeks and 1.16 fold (P<0.001) and 1.30 fold (0.001) after 12 weeks. The 1.11 fold increase noted in Group C was, however, insignificant.

**Effect of Aqueous Infusion of Saffron on CAT**

Activity of the enzyme catalase was also noted to be increased in Group A by 1.26 fold (P<0.01) and in Group B by 1.40 (P<0.001) after treatment with saffron for 2 weeks in comparison with the carcinogen control group. After 12 weeks the enhancement was 1.15 fold (P<0.01) and 1.20 fold (P<0.001) (Figs 9 &10).

**Effect of Aqueous Infusion of Saffron on SOD**

Enhancement of SOD activity was significant in Group A by 1.13 fold (P<0.05) and in Group B by 1.16 fold (P<0.01) following treatment for 2 weeks but only significant in Group B observed after 12 weeks.

**Discussion**

A wide variety of naturally occurring substances including spices (Unnikrisnan, et al. 1990) have been shown to inhibit chemical carcinogenesis in animal models (Williams 1984, Wattenberg 1985, Boone et al. 1999). Saffron, harvested from the dried dark red stigma of Crocus sativus L. flowers is used as a spice to add flavour to exotic food preparations and also finds mention in Indian system of medicine. Analysis of the chemical composition of saffron revealed more than 150 volatile and several non-volatile compounds of which approximately 40-50 constituents have been already identified. (Zargan et al. 1971, Sujata et al. 1992, Tarantilis et al. 1997, Lozano et al. 1999). It has been reported that Saffron can inhibit formation of new tumours as well as reduce or shrink existing tumors. (Nair et al. 1991, 1992, 1994).

In the present report we have demonstrated a chemopreventive effect of saffron on DMBA-induced skin carcinogenesis in female Swiss albino mice. Oral administration of aqueous infusion of saffron was found to produce a remarkable reduction in the incidence of mouse skin papilloma which is a pre-neoplastic benign growth. Topical application of saffron extract was also reported to inhibit skin carcinogenesis and oral administration of saffron extract in the same dose reduced 20-methylcholanthrene (MCA) induced soft tissue sarcoma in mice (Salomi, et al.,1990; 1991). The anti-tumour and anti-carcinogenic effect of saffron may be attributed to presence of a number of biologically active carotenoids like crocin, crocetin and picrocrocin in saffron (Abdullaev 2002). Free radicals and lipid peroxidation are known to cause initiation and promotion of carcinogenesis (Bauer et al. 1980). Lipid peroxidation is increased during the carcinogenic process (Hamburger et al., 1972) and malondialdehyde (MDA) a product of lipid peroxidation was observed to be mutagenic and carcinogenic (Yay et al., 1979. Apaja, 1980, Basu et al., 1983). It is therefore implied that agents that can reduce generation of free radicals in vivo may be considered to have the potential for chemoprevention. The present study shows that saffron treatment could significantly reduce lipid peroxidation in mice exposed to DMBA and croton oil.

GST is thought to play a physiological role in initiating the detoxification of many alkylating agents (Wood, 1970) and environmental chemicals including mutagens and carcinogens (Kramer et al., 1988). Their main function is the conjugation of GSH to a variety of electrophilic compounds. GST reduced the covalent binding of epoxides of carcinogens with DNA and other macromolecules and this reduction in DNA binding was found to be effective in decreasing carcinogenesis caused by the carcinogens. GPx also plays important roles in cellular defence as well as maintenance of cellular membranes from oxidative damage of free radicals by eliminating H2O2 (Sunde et al. 1980).

CAT is a major peroxisomal enzyme. Peroxisomes are involved in a number of important cellular metabolic processes as well as detoxication of H2O2 (Singh et al.,1997; Moser et al. 1996). More than 90% of oxygen consumed by the mitochondria is converted to water (H2O) and the rest to super oxide radical (O2-) whereas the oxygen consumed by peroxisomes is converted to H2O, and only a little amount is converted to O2 (Singh et al., 1996) These reactive oxygen species (ROS) are the normal by-products of cellular metabolism and are checked by cellular defences provided by antioxidant enzymes (Singh et al., 1996). These antioxidant enzymes provide protection to cell against ROS i.e. O2 and H2O2 by detoxifying them at the site where they are produced. So increased activity of GST, GPx and CAT in liver tissue from the mice treated with saffron and decreased activity of these enzymes in the liver tissue from the mice treated with DMBA/croton oil suggested that saffron could influence host detoxification processes. SOD detected in a large number of tissues and organs, is also thought to protect cells from damage by superoxide radicals (Ilan et al. 1981). Superoxide radical O2- is the product of biological oxygen reduction as a result of electron transfer oxidase system. SOD catalyzes the one electron reduction of oxygen to superoxide radical. This O2- is harmful to the respiring cells. SOD minimises the toxicity resulting from the mechanism against oxygen formation of free radicals (Ilan et al. 1981). Increased activity of SOD in the tissue of mice liver treated with saffron suggests certain role for reactive free oxygen radical in two stage carcinogenesis (Werts et al. 1986, Nishuira et al. 1992). Interestingly saffron was found to activate this enzyme also, which was depressed following carcinogen application, to almost near normal values or exceeded the normal values. The group receiving treatment that was started before initiation with DMBA and continued throughout the experimental period produced the best effect.

The increased activities of the Phase II detoxication enzymes noted in this study was accompanied by lowered lipid peroxidation in the liver and reduced incidence of papillomas. The experimental observation therefore suggest
a protective role of saffron against carcinogenic exposure by their action on the physiological detoxification processes. Further investigation using individual chemical compounds present in saffron singly or in combination in a number of in vivo carcinogenesis model would be required to establish the anti-carcinogenic and chemopreventive role of saffron.

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