RESEARCH ARTICLE

Saffron Reduction of 7,12-Dimethylbenz[a]anthracene-induced Hamster Buccal Pouch Carcinogenesis

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Abstract

Our aim was to investigate the chemopreventive potential of saffron in DMBA-induced hamster buccal pouch carcinogenesis. Assessment was by monitoring the percentage of tumor bearing hamsters, tumor size as well as the status of detoxification agents, lipid peroxidation and antioxidants. Oral squamous cell carcinomas were induced in the buccal pouch of Syrian golden hamsters by painting them with 0.5% DMBA in liquid paraffin three times a week for 14 weeks. We observed 100% oral tumor formation with severe histopathological abnormalities in all the hamsters treated with DMBA alone, activities of phase I and phase II detoxification enzymes, lipid peroxidation and antioxidants being significantly altered. Though oral administration of saffron completely prevented the formation of tumors, we noticed severe hyperplasia and dysplasia in hamsters treated with DMBA, suggesting that tumors might eventually develop. Oral administration of saffron return detoxification enzymes, lipid peroxidation and antioxidants to normal ranges. The chemopreventive potential of saffron thus is likely due to antioxidant properties and modulating effects on detoxification in favour of the excretion of carcinogenic metabolites during DMBA-induced hamster buccal pouch carcinogenesis.

Keywords: Antioxidants - chemoprevention - detoxification enzymes - oral cancer - saffron

Asian Pacific J Cancer Prev, **14 (2)**, 951-957

Introduction

Oral cancer, one of the most common malignant neoplasms worldwide, starts as an uncontrolled growth of cells in the mouth and leads to disfigurement of the face, debility of body and eventually death. Oral carcinoma is preceded by clinically identifiable alterations of the oral mucosa, which include erythroplakia and leukoplakia. Of various histological types of oral cancers, over 90% of tumors arising from the oral cavity are found to be squamous cell carcinomas. It has been reported that oral cancer is responsible for highest mortality ratios among all malignancies (Warnakulasuriya et al., 2007).

Epidemiological, experimental and clinical studies pointed out that tobacco smoking, smokeless tobacco products, alcohol consumption, HPV infections, betel quid chewing and low fruits and vegetables intake are the risk factors of oral cancer (Garrote et al., 2001; Massoro et al., 2006). Oral cancer patients, due to asymptomatic nature of oral cancer at initial stages, typically seek medical attention when the malignancy is at an advanced stage. The low five year survival rate of oral cancer patients is also due to late diagnosis, despite recent advancement in oral cancer treatment. Oral cancer can be treated well, if it is diagnosed at early stages (Seoane et al., 2006).

Oral squamous cell carcinoma is the fifth most frequent neoplasm and accounts for over 5,000,000 new cases annually worldwide (Warnakulasuriya, 2009). In USA, approximately 35,000 Americans are affected by oral cancer each year and about 8,000 deaths due to this cancer are reported every year (Stahl et al., 2004). Also, annual increase in oral cancer incidence was reported in Australia, Sri Lanka and Bangladesh (Warnakulasuriya, 2009). In UK and Canada, 3,000-4,000 peoples are affected every year by oral cancer (Parkin et al., 2000). Oral cancer is the most common cancer in India, where oral cancer represents up to 40-50% of all cancers, compared to just 3-4% in the Western countries (Ramadas et al., 2008).

7,12-dimethylbenz[a]anthracene (DMBA), a potent procarcinogen, is commonly employed to induce oral cancer in hamsters, due to the fact that DMBA-induced oral cancer in the buccal pouches of hamsters closely mimics the human oral tumor, both histologically, morphologically and at molecular level. DMBA mediates carcinogenesis in hamsters by inducing chronic inflammation, generating excess reactive oxygen species,

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impairing antioxidant defense mechanism and affecting phase I and II detoxification agents (Manoharan et al., 2010).

Chemoprevention is a useful approach to find out the anti-tumor initiating or anti-tumor promoting potential of natural products and synthetic agents. A large number of oral cancer chemopreventive agents were reported against 7,12-dimethylbenz(a)anthracene (DMBA)-induced oral carcinogenesis (Baskaran et al., 2012; Manoharan et al., 2012a; 2012b; Prabhakar et al., 2012).

Saffron is a naturally derived plant product from the stigma of the Crocus sativus flower. It contains more than 150 volatile, non-volatile and aroma yielding compounds, including safranal, zeaxanthin, lycopene, α- and β-carotenes and carotenoids. Chemical composition analyses have revealed that saffron is composed of approximately 10% moisture, 12% protein, 5% fat, 5% minerals, 5% crude fibre, and 63% sugars including starch, reducing sugars, pentosans, gums, pectin, and dextrins (% w/w) (Abdullaev, 1993). Saffron has traditionally been considered as anti-depressant, respiratory decongestant, anti-spasmodic, aphrodisiac, diaphoretic, emmenagogue, expectorant and sedative (Akhondzadeh et al., 2007). It was used in folk remedy against scarlet fever, small pox, cold, asthma, heart disease and cancer. It has been demonstrated that saffron extract has antitumor and hypolipidemic effects as well as radical scavenging and memory-improving properties (Abdullaev, 2002). It has the ability to reduce inflammation, prevent liver and spleen enlargement, restrict urinary bladder and kidney infections (Premkumar et al., 2006). It has anti-mutagenic, anti-genotoxic, anti-convulsant, anti-nociceptive, antioxidant and cytotoxic effects (Hosseinzadeh et al., 2006). There were however no scientific studies on the chemopreventive potential of saffron in DMBA-induced hamster buccal pouch carcinogenesis. The present study was thus designed to investigate the chemopreventive potential of saffron in DMBA-induced hamster buccal pouch carcinogenesis.

Materials and Methods

Chemicals

7,12-dimethylbenz(a)anthracene (DMBA), saffron and other biochemicals such as reduced glutathione, reduced nicotinamide adenine dinucleotide, 1,1',3,3'-tetramethoxypropane, were obtained from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India. All other biochemicals and reagents were purchased from Hi-media Laboratories Mumbai, India.

Animals

Male golden Syrian hamsters, 8-10 weeks old, weighing 80-120 g were purchased from National Institute of Nutrition, Hyderabad, India and were maintained in Central Animal House, Rajah Muthaiah Medical College and Hospital, Annamalai University. The hamsters were housed in polypropylene cages and provided standard pellet diet and water ad libitum. The hamsters were maintained under controlled conditions of temperature (22±2ºC) and humidity (55±5%) with a 12 h light/dark cycle. Institutional animal ethics committee (Register number 160/1999/CPCSEA), Annamalai University, Annamalai Nagar, India, approved the experimental design (Proposal No. 812 dated: 20-04-2011).

Preparation of aqueous extract of saffron

Saffron was soaked in double distilled water for one hour and homogenized. The homogenate was centrifuged at 2000 rpm for 10 min and the supernatant was used to assess the chemopreventive potential. The dose of 100 mg/kg bw was chosen for chemoprevention studies, based on previous literatures (Premkumar et al., 2006).

Induction of oral squamous cell carcinoma

Oral squamous cell carcinoma was developed in the buccal pouch of Syrian golden hamsters by painting with 0.5% 7,12-dimethylbenz[a]anthracene (DMBA) in liquid paraffin three times a week for 14 weeks.

Experimental design

A total of 24 hamsters were randomized into four groups of six hamsters in each. Group I hamsters served as control and were painted with liquid paraffin alone three times a week for 14 weeks on their left buccal pouches. Groups II and III hamsters were painted with 0.5% DMBA in liquid paraffin three times a week for 14 weeks on their left buccal pouches. Group II hamsters received no other treatment. Group III hamsters were orally given saffron at a dose of 100 mg/kg bw/day, starting one week before exposure to the carcinogen and continued on days alternate to DMBA painting, until the sacrification of the hamsters. Group IV hamsters received oral administration of saffron alone (100 mg/kg bw) throughout the experimental period. Every day the hamsters were treated with either DMBA or saffron between 8.00 am and 9.00 am. The experiment was terminated at the end of 16th week and all hamsters were sacrificed by cervical dislocation.

Histological evaluation

For histopathological examination, buccal mucosal tissues were fixed in 10% formalin and routinely processed and embedded with paraffin, 2-3 μm sections were cut in a rotary microtome and stained with haematoxylin and eosin.

Biochemical estimations

Biochemical studies were conducted on plasma, liver and buccal mucosa of control and experimental hamsters in each group. The status of phase I (cytochrome P450 and b5) and phase II (GR, GST, GSH and DT-diaphorase) detoxification agents, lipid peroxidation byproduct (TBARS) and antioxidants (SOD, CAT, GPx, GSH and vitamin E) was assayed using specific and sensitive colorimetric methods.

The levels of cytochrome P450 and b5 in the liver and buccal mucosa were determined according to the method of Omura and Sato (1964). The activity of glutathione-S-transferase in liver and buccal mucosa tissue homogenate was assayed using the method employed by Habig et al. (1994). Glutathione reductase activity in liver tissue homogenate was assayed using the

method employed by Carlberg and Mannervik (1985). The activity of DT-diaphorase in the liver was estimated according to the method of Ernster (1967). Lipid peroxidation was estimated as evidenced by the formation of thiobarbituric acid reactive substances. Thiobarbituric acid reactive substances in plasma were assayed by the method described by Yagi (1987). Buccal mucosa lipid peroxidation was done using the method employed by Ohkawa et al. (1979). Superoxide dismutase activity was assayed in plasma and buccal mucosa using the method employed by Kakkar et al. (1985). The activity of catalase in plasma and buccal mucosa was assayed using the method described by Sinha (1972). The activity of glutathione peroxidase in plasma and buccal mucosa was determined using the method employed by Rotruck et al. (1973). The reduced glutathione levels in the plasma and buccal mucosa were determined by the method described by Beutler and Kelly (1963). The vitamin E level in the plasma was determined colorimetrically using the method described by Palan et al. (1973). Buccal mucosa vitamin E was measured using the fluorimetric method described by Desai (1984).

Statistical analysis

The values are expressed as mean±SD. The statistical comparisons were performed by one way analysis of variance (ANOVA) followed by (DMRT) Duncan's Multiple Range Test using SPSS version 16.0 for windows (SPSS Inc. Chicago; http://www.spss.com). The values were considered statistically significant if the p-values were less than 0.05.

Results

We observed 100% tumor formation with mean tumor volume (194.27±12.4 mm^3) and tumor burden (583.08±36.4 mm^3) in hamsters treated with DMBA alone and the tumors were histopathologically confirmed as well differentiated squamous cell carcinoma. Also, we noticed severe keratoses, hyperplasia and dysplasia in hamsters treated with DMBA alone (Figure 1B). Though oral administration of saffron at a dose of 100 mg/kg bw completely prevented the tumor incidence, severe hyperplasia and dysplasia were noticed in hamsters treated with DMBA (Figure 1C). A well defined intact epithelium was noticed in hamsters treated with saffron alone (Figure 1D) and control hamsters (Figure 1A).

Figure 2 shows the status of phase I (cytochrome P450 and b5) and phase II (GR, GST, GSH and DT-diaphorase) detoxification agents in the liver of control and experimental hamsters in each group. The status of phase I detoxification agents was significantly increased whereas phase II agents were decreased in the liver of DMBA treated hamsters as compared to control

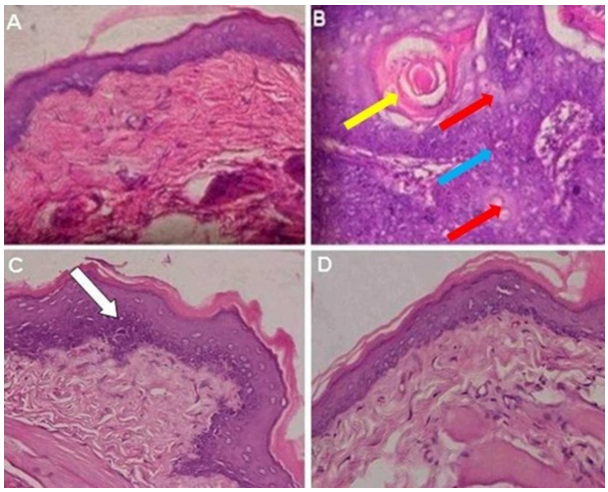

Figure 1. Histopathological Changes in the Buccal Mucosa of Control and Experimental Hamsters in Each Group. (A & D): Photomicrographs showing well-defined buccal pouch epithelium from control and saffron alone treated hamsters respectively (H & E, 40X). (B): Photomicrographs showing well-differentiated squamous cell carcinoma with keratin pearls (--->), hyperplasia (--->) and dysplasia (--->) in hamsters treated with DMBA alone (H & E, 40X). (C): Photomicrographs showing dysplastic epithelium (--->) in hamsters treated with DMBA + Saffron (H & E, 40X)

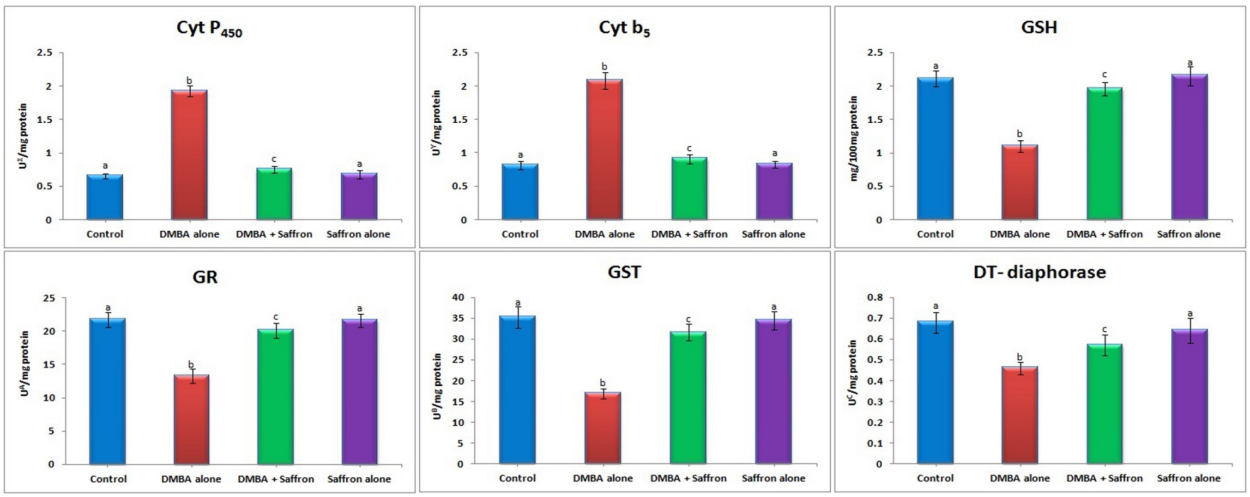

Figure 2. Status of Phase I and Phase II Detoxification Agents in the Liver of Control and Experimental Hamsters in Each Group. Values are expressed as mean±SD (n=6). Values that are not sharing a common superscript letter between groups differ significantly at $p<0.05$. X - Micromoles of cytochrome P450 formed; Y - Micromoles of cytochrome b5 formed; A-Micromoles of NADPH oxidized per minute; B-Micromoles of CDNB-GSH conjugate formed per minute; C-Micromoles of 2,6-dichlorophenol reduced per minute

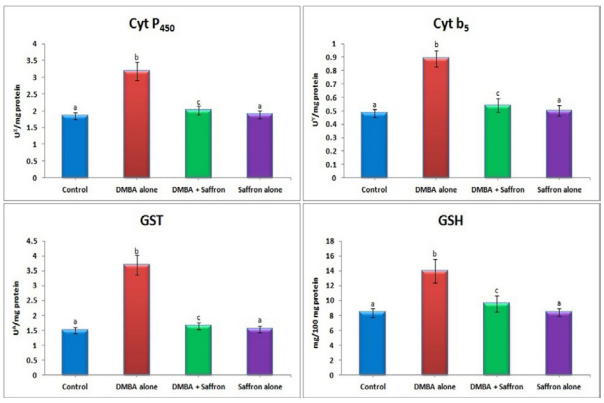

Figure 3. Status of Phase I and Phase II Detoxification Agents in the Buccal Mucosa of Control and Experimental Hamsters in Each Group. Values are expressed as mean±SD for 6 hamsters in each group. Values that do not share a common superscript between groups differ significantly at p<0.05. X-Micromoles of cytochrome P450 formed; Y-Micromoles of cytochrome b5 formed; A-Micromoles of 1-chloro-2,4-dinitrobenzene (CDNB)/reduced glutathione conjugate formed per minute

hamsters. Oral administration of saffron to DMBA treated hamsters brought back the status of phase I and phase II detoxification agents to near normal range in the liver. Oral administration of saffron alone showed no significant difference in the status of phase I and II detoxification agents as compared to control hamsters.

Figure 3 shows the status of phase I (cytochrome P450 and b5) and phase II (GST and GSH) detoxification agents in the buccal mucosa of control and experimental hamsters in each group. The status of phase I (cytochrome P450 and b5) and phase II detoxification agents (GST and GSH) were significantly increased in tumor bearing hamsters as compared to control hamsters. Oral administration of saffron to DMBA treated hamsters brought back the status of phase I and phase II detoxification agents to near normal range in the buccal mucosa. Oral administration of saffron alone, showed no significant difference in the status of phase I and II detoxification agents as compared to control hamsters.

Figure 4 shows the status of plasma TBARS and antioxidants (SOD, CAT, GPx, GSH and vitamin E)

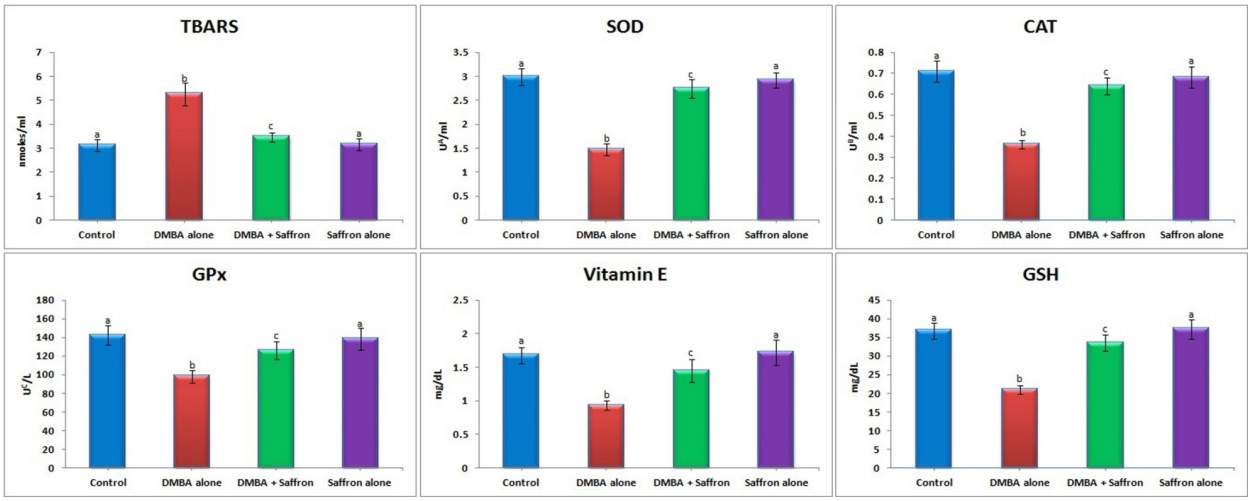

Figure 4. Status of TBARS and Antioxidants in the Plasma of Control and Experimental Hamsters in Each Group. Values are expressed as mean±SD for 6 hamsters in each group. Values that do not share a common superscript between groups differ significantly at p<0.05. A-the amount of enzyme required to inhibit 50% NBT reduction; B-micromoles of hydrogen peroxide utilized/s; C-micromoles of glutathione utilized/min

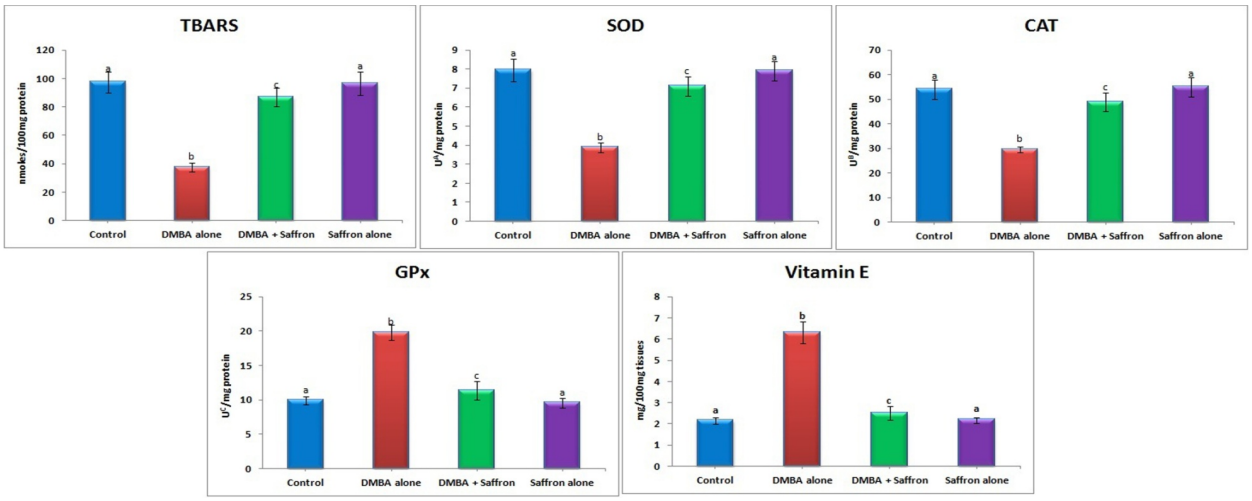

Figure 5. Status of TBARS and Antioxidants in the Buccal Mucosa of Control and Experimental Hamsters in Each Group. Values are expressed as mean±SD for 6 hamsters in each group. Values that do not share a common superscript between groups differ significantly at p<0.05. A-the amount of enzyme required to inhibit 50% NBT reduction; B-micromoles of hydrogen peroxide utilized/sec.; C-micromoles of glutathione utilized/min

in control and experimental hamsters in each group. The concentration of TBARS was increased whereas antioxidants activities were decreased in DMBA treated hamsters as compared to control hamsters. Oral administration of saffron at a dose of 100 mg/kg bw restored the concentration of TBARS and antioxidants to near normal range in DMBA treated hamsters. Hamsters treated with saffron alone showed no significant difference in TBARS and antioxidants status as compared to control hamsters.

Figure 5 shows the status of buccal mucosa TBARS and antioxidants (SOD, CAT, GPx and vitamin E) in control and experimental hamsters in each group. The concentration of TBARS and activities of SOD and CAT were decreased whereas the status of GPx and vitamin E were increased in DMBA treated hamsters as compared to control hamsters. Oral administration of saffron at a dose of 100 mg/kg bw restored the concentration of TBARS and antioxidants in DMBA treated hamsters to near normal range. Hamsters treated with saffron alone showed no significant difference in TBARS and antioxidants status as compared to control hamsters.

Discussion

Oral carcinogenesis has multifactorial etiology and usually preceded by distinct premalignant lesions such as leukoplakia and erythroplakia. DMBA-induced hamster buccal pouch carcinogenesis is an accepted experimental animal model to test the chemopreventive potential of natural products and synthetic agents. Medicinal plants and their bioactive constituents exert their chemopreventive efficacy by modulating phase I and II detoxification cascade and improving antioxidant defense mechanism (Priyadarsini et al., 2009).

In the present study, the chemopreventive potential of saffron was assessed in DMBA-induced hamster buccal pouch carcinogenesis, by monitoring the percentage of tumor bearing hamsters and tumor size as well as by analyzing the status of detoxification agents, lipid peroxidation and antioxidants. We observed 100% tumor formation in the hamster buccal pouches treated with DMBA alone. The tumor excised from the buccal pouches was subjected to histopathological studies. The oral pathologist confirmed the tumor as well differentiated squamous cell carcinoma. The tumor tissues showed pleomorphic hyperchromatic nuclei with epithelial pearl formation. The tumor tissues also revealed severe keratosis, hyperplasia and dysplasia. Oral administration of saffron completely prevented the tumor formation in DMBA treated hamsters. A mild to moderate preneoplastic lesions such as hyperplasia, keratosis and dysplasia were however noticed in DMBA + saffron treated hamsters. Our results thus suggest that saffron might have suppressed abnormal cell proliferation during DMBA-induced oral carcinogenesis.

Phase I (cytochrome P450 and b5) and II (GR, GST, GSH and DT-diaphorase) biotransformation enzymes play pivotal role in the metabolic activation and excretion of carcinogens and their metabolites, respectively. Any impairment in the activities of these enzymes could result in malignant transformation, due to accumulation of carcinogenic metabolites (Manoharan et al., 2010). In the present study, the status of phase I and II detoxification agents were altered in the liver and buccal mucosa of hamsters treated with DMBA alone. Our results thus suggest that the activities of detoxification cascade was significantly impaired due to repeated exposure to DMBA, which resulted in the accumulation of the carcinogenic metabolite, dihydrodiol epoxide. Oral administration of saffron to hamsters treated with DMBA restored the status of liver and buccal mucosa phase I and II detoxification agents. Our results thus suggest that saffron modulated the activities of phase I and II detoxification enzymes in favour of the excretion of the carcinogenic metabolites of DMBA.

Over production of lipid peroxidation by-products in the cell can make them weak and defenseless. Measurement of plasma lipid peroxidation by-products helps to assess the extent of tissue damage. Extensive studies suggested that lipid peroxides that are generated excessively at the primary site could be transferred through circulation to other organs and provoke damage by propagating lipid peroxidation (Dhanarasu et al., 2010). Increased plasma TBARS observed in the plasma of hamsters treated with DMBA alone is probably due to over production and diffusion of lipid peroxidation by-products from the damaged host tissues with subsequent leakage into plasma (Senthil et al., 2007). Lowered levels of non-enzymatic antioxidants (vitamin E and GSH) and decreased activities of enzymatic antioxidants (SOD, CAT and GPx) could be responsible for increased plasma TBARS in hamsters treated with DMBA alone. Our results suggest that the antioxidant defense system was significantly impaired during DMBA-induced oral carcinogenesis. Decreased levels of vitamin E and GSH content in plasma are probably due to their utilization by tumor tissues to meet their nutrient demand for the rapid growth (Vinothkumar et al., 2011). Lowered activities of SOD, CAT and GPx in plasma are probably due to exhaustion of these enzymes to scavenge excessively generated reactive oxygen species in the system (Manoharan et al., 2009).

In the present study, we observed low levels of buccal mucosa TBARS and disturbed antioxidant status in hamsters treated with DMBA alone. Low levels of lipid peroxidation by-products were reported in highly proliferating malignant tumors. Low levels of PUFA in tumor tissues were reported as responsible factor for decreased levels of lipid peroxides in oral carcinoma (Kavitha et al., 2006). Our results corroborate these observations. GPx and GSH have regulatory effect on cell proliferation. Increase in GPx activity and GSH content in the buccal mucosa of hamsters treated with DMBA alone support the above findings (Manikandan et al., 2008). Lowered activities of SOD and CAT were reported in the tumor tissues of several cancerous conditions (Upadhya et al., 2004). Our results are in line with these findings. Oral administration of saffron restored the status of lipid peroxidation and antioxidants in the plasma and buccal mucosa of DMBA treated hamsters. Our results thus suggest that saffron showed potent antilipid peroxidative and antioxidant function during DMBA-induced oral

carcinogenesis.

The present study thus demonstrates the chemopreventive potential of saffron in DMBA-induced hamster buccal pouch carcinogenesis. The chemopreventive potential of saffron is probably due to its anti-lipid peroxidative or antioxidant potential and modulating effect on phase I and II detoxification cascade in favour of the excretion of carcinogenic metabolite.

In conclusion, in the present study, although saffron completely prevented the tumor formation in hamsters treated with DMBA, we however noticed severe hyperplasia and dysplasia after 14 weeks. The present study should therefore be extended to confirm whether saffron delayed the tumor formation or inhibited the tumor formation in hamsters treated with DMBA. The present study will be extended in future to assess the molecular chemopreventive potential of saffron by analyzing the expression pattern of cell proliferative, apoptotic, inflammatory and angiogenic markers in the buccal mucosa of hamsters treated with DMBA.

Acknowledgements

The authors gratefully acknowledge Dr. Madavan R Nirmal, Professor, Department of Oral Pathology, and Mr. P. Rajamanikam, Lab technician, Department of Biochemistry for their valuable assistance in histopathological studies and animal sacrification.

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