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Protective Effect of *Crocus sativus* Stigma Extract and Crocin (*trans*-crocin 4) on Methyl Methanesulfonate–Induced DNA Damage in Mice Organs

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This study was designed to examine the effect of aqueous extract of *Crocus sativus* stigmas (CSE) and crocin (*trans*-crocin 4) on methyl methanesulfonate (MMS)–induced DNA damage in multiple mice organs using comet assay. Adult male NMRI mice in different groups were treated with either physiological saline (10 mL/Kg, intraperitoneal [ip]), CSE (80 mg/Kg, ip), crocin (400 mg/Kg, ip), MMS (120 mg/Kg, ip), and CSE (5, 20, and 80 mg/Kg, ip) 45 min prior to MMS administration or crocin (50, 200, and 400 mg/Kg, ip) 45 min prior to MMS administration. Mice were scarified about 3 h after each different treatment, and the alkaline comet assay was used to evaluate the effect of these compounds on DNA damage in different mice organs. The percent of DNA in the comet tail (% tail DNA) was measured. A significant increase in the % tail DNA was seen in nuclei of different organs of MMS-treated mice. In control groups, no significant difference was found in the % tail DNA between CSE- or crocin-pretreated and saline-pretreated mice. The MMS-induced DNA damage in CSE-pretreated mice (80 mg/Kg) was decreased between 2.67-fold (kidney) and 4.48-fold (lung) compared to those of MMS-treated animals alone ($p < 0.001$). This suppression of DNA damage by CSE was found to be depended on the dose, which pretreatment with CSE (5 mg/Kg) only reduced DNA damage by 6.97%, 6.57%, 7.27%, and 9.90% in liver, lung, kidney, and spleen, respectively ($p > 0.05$ as compared with MMS-treated group). In the same way, crocin also significantly decreased DNA damage by MMS (between 4.69-fold for liver and 6.55-fold for spleen, 400 mg/Kg), in a dose-dependent manner. These data indicate that there is a genoprotective property in CSE and crocin, as revealed by the comet assay, *in vivo*.

Introduction

Considerable amount of epidemiological evidence and laboratory investigations has shown a protective effect of diets rich in fruits and vegetables against cancer by inhibiting genotoxins and/or carcinogens through the biologically active plant secondary metabolites (Rogers *et al.*, 1993; Ferguson, 1994; Ames, 2001). Therefore, assessment of their potential and understanding their probable mechanisms of action could be useful for better management of chemopreventive strategies.

Crocus sativus L., commonly known as saffron, is a perennial stemless herb that is widely cultivated in different parts of the world, especially Greece, India, Iran, and Spain. The major biologically active ingredients of saffron are crocins (which are glycoside derivatives of *trans*-crocetin), picrocrocin, and safranal (Tarantilis *et al.*, 1995). Saffron is used in folk medicine as an anodyne, antidepressant, sedative, respiratory decongestant, anticatarrhal, expectorant, antispasmodic, eupeptic, stomachic, carminative, diaphoretic, gingival sedative, aphrodisiac, and emmenagogue (Rios *et al.*, 1996; Abdullaev and Espinosa-Aguirre, 2004). Further, modern pharmacological studies have shown that saffron extract or its active constituents have learning- and memory-improving properties (Abe and Saito, 2000; Pitsikas *et al.*, 2007), anticonvulsant (Hosseinzadeh and Khosravan, 2002; Hosseinzadeh and Talebzadeh, 2005), antidepressant (Hosseinzadeh *et al.*, 2004; Akhondzadeh *et al.*, 2004), antiinflammatory (Hosseinzadeh and Younesi, 2002), antiischemic (Hosseinzadeh and Sadeghnia, 2005; Hosseinzadeh *et al.*, 2008; Zheng *et al.*, 2007), and antitumor effects (Abdullaev, 1993, 2002; Abdullaev and Espinosa-Aguirre, 2004). Radical scavenging, antioxidant activity, and promotion of the diffusivity of oxygen in different tissues were also reported for saffron extract or its bioactive constituents (Rios *et al.*, 1996; Verma and Bordia, 1998; Assimopoulou *et al.*, 2005; Kanakis *et al.*, 2007a). Saffron extract also has chemopreventive and genoprotective effects and protects from genotoxin-induced oxidative stress in mice (Nair *et al.*, 1995;

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Premkumar *et al.*, 2001; Abdullaev *et al.*, 2002; Premkumar *et al.*, 2003).

Recently, we have found that safranal, a constituent of saffron, could exert protective effect against methyl methanesulfonate (MMS)–induced DNA damage in mice organs (Hosseinzadeh and Sadeghnia, 2007). In this study we designed to examine the influence of aqueous extract of *C. sativus* stigmas (CSE) and crocin (*trans*-crocin 4), another constituent of saffron, on MMS-induced DNA damage in multiple mice organs (liver, lung, kidney, and spleen), using alkaline single gel electrophoresis (SCGE) or comet assay.

Materials and Methods

Animals

Adult male NMRI mice weighing 25–30 g were used throughout the study. All of them were kept in the same room under a constant temperature (22 ± 2°C) and illuminated 7:00 a.m. to 7:00 p.m., with food pellets and water available *ad libitum*. The experiment was approved by the University's Ethics Committee for Animal Use.

Chemicals

Chemicals were obtained from the following sources: low melting point (LMP) agarose from Biogen (Mashhad, I.R. Iran); normal melting point (NMP) agarose from Fermentas (Glen Burnie, MD); crocin (*trans*-crocin 4) from Fluka (St. Gallen, Switzerland); and sodium hydroxide (NaOH), sodium chloride (NaCl), ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), Tris (hydroxymethyl) aminomethane (Trizma base), *t*-octylphenoxypoly-ethoxyethanol (Triton X-100), dimethylsulfoxide (DMSO), sodium lauroylsarcosinate (sarkosyl, SLS), MMS, and methanol from Merck (Darmstadt, Germany). LMP and NMP agarose were diluted in physiological saline to 0.5% and 1%, respectively.

Preparation of aqueous saffron extract

C. sativus L. stigmas were collected from Ghaen (Khorasan Province, northeast of Iran). In the maceration method, 1 g of stigma was macerated in 800 mL distilled water and gently shaken for 3 days. The mixture was subsequently filtered, and concentrated under reduced pressure at 35°C. The extract contains 1.97% of *trans*-crocin 4 (Modaghegh *et al.*, 2008).

Treatment and organ preparation

Ten groups of male mice were used for this experiment (for each treatment group, $n = 5–7$). The animals in different groups were received the following chemicals: physiological saline (10 mL/Kg, ip), CSE (80 mg/Kg, ip), crocin (400 mg/Kg), MMS (120 mg/Kg, ip), CSE (5, 20, and 80 mg/Kg, ip) 45 min prior to MMS administration, and crocin (50, 200, and 400 mg/Kg, ip) 45 min prior to MMS administration. About 3 h after injection, the animals were killed by cervical dislocation, and four organs (liver, lung, spleen, and kidney) were removed. Changes in size, color, and texture of organs were examined. After weighing the organs, they were minced, suspended at a concentration of 0.5 g/mL in chilled homogenizing buffer containing 0.075 M NaCl and 0.024 M Na_2EDTA (pH 7.5), and then homogenized gently at 500–800 rpm in ice. To obtain nuclei, the homogenate was centrifuged at 700 *g* for 10 min at 0°C, and the precipitate was resuspended in chilled homogenizing buffer at 0.5 g/mL and allowed to settle; precipitated clumps were then removed. Doses and times were selected based on the preliminary studies as well as literature-reported values (Sasaki *et al.*, 1997a, 1997b; Tsuda *et al.*, 2000; Sekihashi *et al.*, 2002).

Slide preparation and alkaline SCGE assay

The *in vivo* alkaline SCGE assay was conducted based on the method described by Sasaki *et al.* (1997a) with some modifications. One hundred microliters of NMP agarose was quickly layered on conventional slides, the slides were covered with a cover slip, and then the slides were placed on ice to allow agarose to gel. Five microliters of the nucleus suspension, prepared as above, was mixed with 75 μL LMP agarose, and the mixture was quickly layered over the NMP agarose layer after removal of the cover slip. Finally, another layer of LMP agarose was added on top. The slides were immersed immediately in a chilled lysing solution (pH 10) made up of 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma, 1% sarkosyl, 10% DMSO, and 1% Triton X-100, and kept at 0°C in the dark overnight. Then, the slides were placed on a horizontal gel electrophoresis platform and covered with a chilled alkaline solution made up of 300 mM NaOH and 1 mM Na_2EDTA (pH 13). They were left in the solution in the dark at 0°C for 40 min, and then electrophoresed at 0°C in the dark for 30 min at 25 V and approximately 300 mA. The slides were rinsed gently three times with 400 mM Trizma solution (adjusted to pH 7.5 by HCl) to neutralize the excess alkali, stained with 50 μL of 20 μg/mL ethidium bromide, and covered with a cover slip.

Examination of the nuclei and statistical analysis

One hundred nuclei per organ from each animal (50 nuclei on one slide) were examined and photographed through a fluorescence microscope (Nikon, Kyoto, Japan) at 400× magnification equipped with an excitation filter of 520–550 nm and a barrier filter of 580 nm.

Undamaged cells resemble an intact nucleus without a tail, and damaged cells have the appearance of a comet. The percent of DNA in the comet tail (% tail DNA), which is an estimate of DNA damage, was measured using a computerized image analysis software (CASP software). Statistical analysis was performed using one-way ANOVA followed by Tukey–Kramer *post hoc* test for multiple comparisons. The *p*-values less than 0.05 were considered to be statistically significant.

Results

In this study, % tail DNA was measured as an indicator of DNA damage. A significant increase in the % tail DNA was seen in nuclei of different organs of MMS-treated mice, as compared to those of saline-treated animals ($p < 0.001$; Figs. 1 and 2). In control groups, no significant difference was found in the % tail DNA between CSE- or crocin-pretreated and saline-pretreated mice. The MMS-induced DNA damage in CSE-pretreated mice (80 mg/Kg) was decreased between 2.67-fold (kidney) and 4.48-fold (lung) as compared to that of MMS-treated animals alone ($p < 0.001$; Fig. 1A–D). This

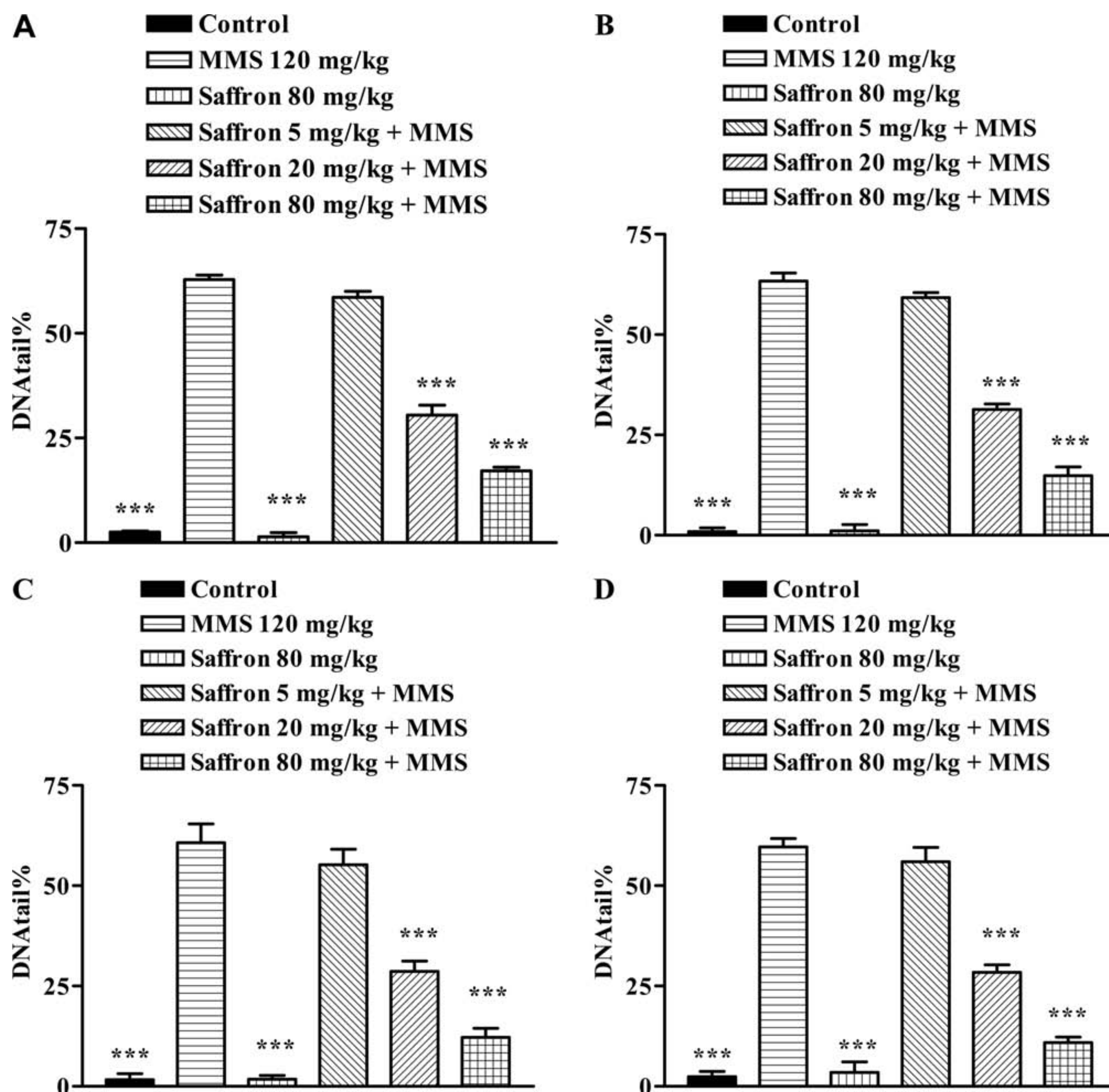

FIG. 1. (**A**) Effect of saffron aqueous extract on DNA damage induced by MMS in mouse liver. CSE was administered 45 min prior to MMS injection, ip. Values are mean ± SEM of 5–7 mice. ***$p < 0.001$ as compared with MMS-treated mice (one-way ANOVA followed by Tukey–Kramer test). (**B**) Effect of saffron aqueous extract on DNA damage induced by MMS in mouse lung. CSE was administered 45 min prior to MMS injection, ip. Values are mean ± SEM of 5–7 mice. ***$p < 0.001$ as compared with MMS-treated mice (one-way ANOVA followed by Tukey–Kramer test). (**C**) Effect of saffron aqueous extract on DNA damage induced by MMS in mouse kidney. CSE was administered 45 min prior to MMS injection, ip. Values are mean ± SEM of 5–7 mice. ***$p < 0.001$ as compared with MMS-treated mice (one-way ANOVA followed by Tukey–Kramer test). (**D**) Effect of saffron aqueous extract on DNA damage induced by MMS in mouse spleen. CSE was administered 45 min prior to MMS injection, ip. Values are mean ± SEM of five mice. ***$p < 0.001$ as compared with MMS-treated mice (one-way ANOVA followed by Tukey–Kramer test).

suppression of DNA damage by CSE was found to be depended on the dose, which pretreatment with CSE (5 mg/Kg) only reduced DNA damage by 6.97%, 6.57%, 7.27%, and 9.90% in liver, lung, kidney, and spleen, respectively ($p > 0.05$ as compared with MMS-treated group; Fig. 1A–D). In the same way, crocin also significantly decreased DNA damage by MMS (between 4.69-fold for liver and 6.55-fold for spleen, 400 mg/Kg), in a dose-dependent manner (Fig. 2A–D).

Discussion

Recently, considerable attention has been focused on the use of natural compounds as chemopreventive/chemoprotective

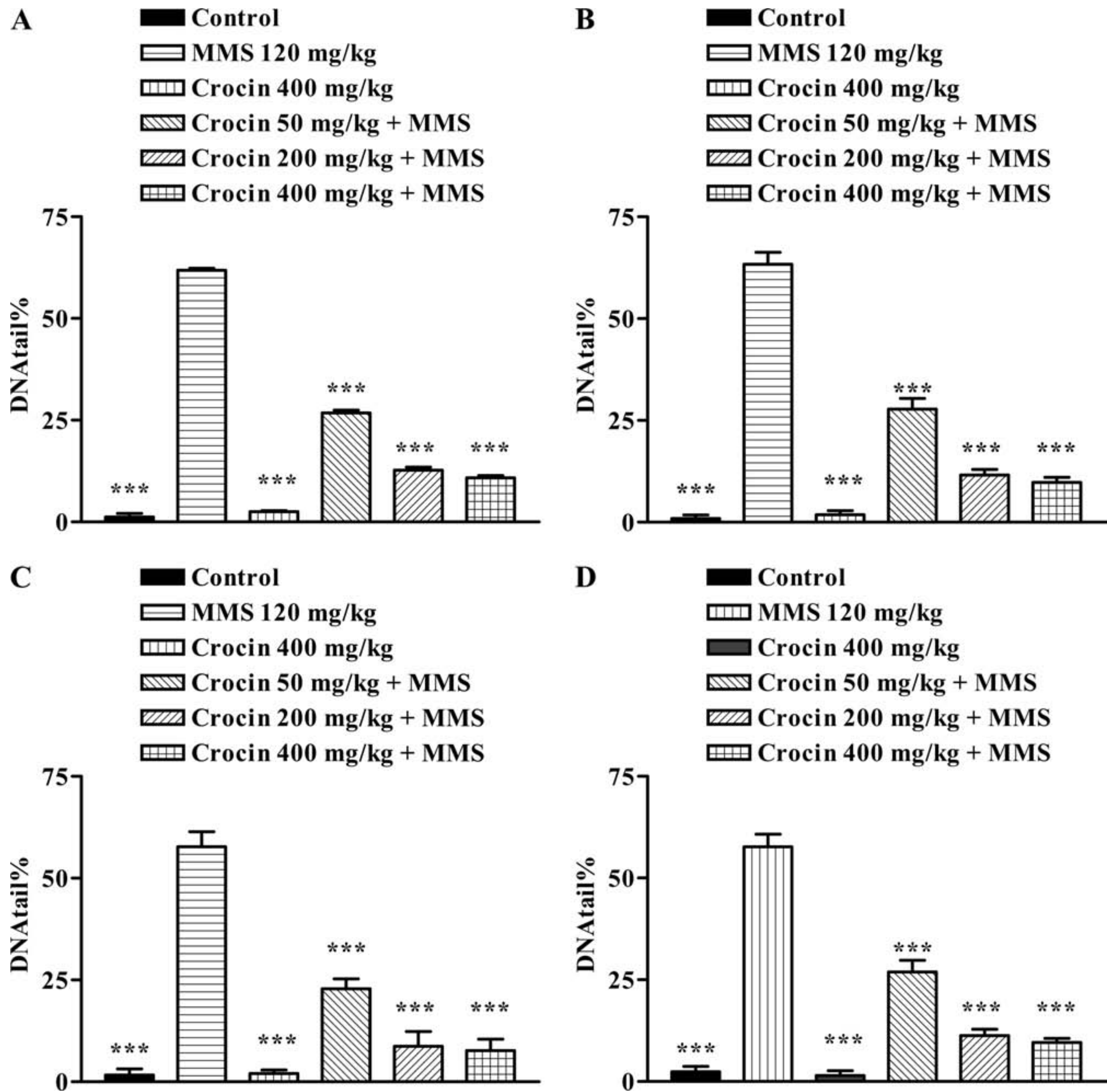

FIG. 2. (**A**) Effect of crocin on DNA damage induced by MMS in mouse liver. Crocin was administered 45 min prior to MMS injection, ip. Values are mean ± SEM of 5–7 mice. ***$p < 0.001$ as compared with MMS-treated mice (one-way ANOVA followed by Tukey–Kramer test). (**B**) Effect of crocin on DNA damage induced by MMS in mouse lung. Crocin was administered 45 min prior to MMS injection, ip. Values are mean ± SEM of 5–7 mice. ***$p < 0.001$ as compared with MMS-treated mice (one-way ANOVA followed by Tukey–Kramer test). (**C**) Effect of crocin on DNA damage induced by MMS in mouse kidney. Crocin was administered 45 min prior to MMS injection, ip. Values are mean ± SEM of 5–7 mice. ***$p < 0.001$ as compared with MMS-treated mice (one-way ANOVA followed by Tukey–Kramer test). (**D**) Effect of crocin on DNA damage induced by MMS in mouse spleen. Crocin was administered 45 min prior to MMS injection, ip. Values are mean ± SEM of five mice. ***$p < 0.001$ as compared with MMS-treated mice (one-way ANOVA followed by Tukey–Kramer test).

agents. Because of a strong correlation between genomic damage and carcinogenesis (Hoeijmakers, 2001), understanding the antigenotoxic potential of these compounds should be useful in cancer chemopreventive therapy.

There are two major findings in our study. First, no considerable DNA damage was seen in mice pretreated with relatively high doses of CSE and crocin, as revealed by comet assay. Second, pretreatment with CSE and crocin significantly suppressed MMS-induced DNA damage in multiple mice organs, in a dose-dependent manner. Although all doses of crocin significantly attenuated DNA damage induced by MMS, there was also a significant difference ($p < 0.001$) between pretreatment with the low dose (50 mg/Kg) and high dose (200 mg/Kg) of crocin, which means that the protective

effect of the treatment with lower doses of crocin against MMS-induced genotoxicity cannot be postulated.

The results of the present study, however, are consistent with previous findings. It has been reported that saffron or its ingredients such as crocin and dimethylcrocin are not mutagenic or genotoxic (Salomi *et al*., 1991a; Abdullaev *et al*., 2002, 2003). Premkumar *et al*. also showed that saffron aqueous extract protects from genotoxicity as well as genotoxins-induced oxidative stress in mice (Premkumar *et al*., 2001, 2003, 2006). In these studies, oral pretreatment with aqueous saffron extract (20, 40, and 80 mg/Kg) for 5 consecutive days significantly inhibited the genotoxicity of antitumor drugs (cyclophosphamide, mitomycin C, and cisplatin), *in vivo*, as revealed by micronucleus and comet assay. It was suggested that saffron could exert its antigenotoxic and chemopreventive effects by the modulation of antioxidants and/or detoxification systems (Premkumar *et al*., 2001, 2006). It was also reported that crocetin could significantly inhibit the genotoxic effects and neoplastic transformations of C3H1OT1/2 cells by benzo(a)pyrene (Chang *et al*., 1996). An inhibitory effect of safranal on MMS-induced genotoxicity has also been shown in multiple mice organs (Hosseinzadeh and Sadeghnia, 2007). Therefore, safranal may also contribute to the preventive effect of saffron against MMS-induced DNA damage.

Several hypotheses may explain the attenuation of MMS-induced genotoxicity by CSE and crocin. A direct interaction with nucleic acids such as DNA and thereby protecting them from harmful damages were reported for crocin, crocetin, dimethylcrocin, and safranal, *in vitro* (Bathaie *et al*., 2007; Kanakis *et al*., 2007b, 2007c). It could be postulated that saffron decreases DNA damage by protecting DNA nucleophilic sites.

It is documented that chemopreventive agents can exert their antimutagenic/anticarcinogenic effects via several mechanism such as inhibiting the carcinogen activation, enhancing detoxification of carcinogenic agents, quenching reactive oxygen species or tapping of electrophiles, inhibition of cell replication, modulation of DNA metabolism and repair, control of gene expression (like inhibition of oncogene expression), influencing of apoptosis, and inhibition of tumor promotion or progression (De Flora, 1998). Among the detoxification mechanisms, glutathione (GSH) and its related enzymes have a central role (Ketterer, 1988; Franco *et al*., 2007). It is generally believed that agents increasing the GSH pool and inducing its related enzymes (such as GSH peroxidase, GSH reductase, and GSH -S-transferase) could inhibit the genotoxicity and carcinogenicity of toxic agents.

The protective effects of saffron and crocin observed in the present study may be related to its antioxidant and radical scavenger properties. Saffron and crocin may also decrease the MMS-induced genotoxicity by enhancing the systems involve in detoxification and mutagen/carcinogen inactivation. It has been shown that antioxidants reduced chemically induced carcinogenesis (Khan *et al*., 2008) and inhibited DNA lesions induced by alkylating agents such as MMS (Kaya, 2003; Arranz *et al*., 2007). Saffron and its carotenoids scavenge free radicals and thereby may protect cells from oxidative stress (Bors *et al*., 1982; Abdullaev, 1993; Nair *et al*., 1995; Rios *et al*., 1996; Pham *et al*., 2000; Assimopoulou *et al*., 2005). Recently, Papandreou *et al*. (2006) showed that CSE and its crocin constituents possess good antioxidant properties, and inhibit amyloid β-peptide (Aβ) fibrils aggregation in a concentration- and time-dependent manner, *in vitro*. Ameliorating effects of saffron on cisplatin-induced toxicity and changes in enzyme activities have also been reported (Nair *et al*., 1991b; El Daly, 1998). It is known that crocins and crocetin decrease the aflatoxin B_1 and dimethylnitrosamine-induced hepatotoxicity and protects rat primary hepatocytes against oxidative damage (Lin and Wang, 1986; Wang *et al*., 1991a, 1991b; Tseng *et al*., 1995).

Reduction of lipid peroxidation, improving of total antioxidant capacity, total thiol contents, and GSH pool, as well as antioxidant enzyme activities such as superoxide dismutase, catalase, and GSH-related enzymes were reported for saffron, crocin, and safranal following ischemia-reperfusion injury (Hosseinzadeh and Sadeghnia, 2005; Hosseinzadeh *et al*., 2005; Saleem *et al*., 2006; Hosseinzadeh *et al*., 2008; Zheng *et al*., 2007). The modifying effects of saffron and its carotenoids on antioxidant enzymatic activities have also been shown in other experimental models (Premkumar *et al*., 2001; Premkumar *et al*., 2003; Shen and Qian, 2006). Moreover, crocin and other carotenoids from saffron prevent the death of rat pheochromocytoma (PC-12) cells, apoptotic morphological changes, and DNA fragmentation induced by tumor necrosis factor (TNF)-α and serum/glucose deprivation by its antioxidant effects and increasing GSH synthesis (Soeda *et al*., 2001; Ochiai *et al*., 2004a, 2004b, 2007).

There are several reports about chemopreventive and tumoricidal properties of saffron or its constituents, *in vivo* and *in vitro* (Salomi *et al*., 1991b; Nair *et al*., 1991a; Nair *et al*., 1994, 1995; Escribano *et al*., 1996; Abdullaev, 2002; Abdullaev *et al*., 2002; Abdullaev and Espinosa-Aguirre, 2004; Das *et al*., 2004; Chryssanthi *et al*., 2007). Abdullaev and Frenkel also showed that saffron could exert inhibitory effect on cellular DNA and RNA synthesis, but not on protein synthesis (Abdullaev and Frenkel, 1992a, 1992b). Cancer chemopreventive as well as antitumor activities were also reported for crocins and crocetin derivatives in different assay systems (Tarantilis *et al*., 1994; Wang *et al*., 1995; Escribano *et al*., 1996; Konoshima *et al*., 1998; Garcia-Olmo *et al*., 1999; Aung *et al*., 2007).

Another possibility is complex formation and inactivation of the alkylating agent by CSE and crocin, which were classified as desmutagenicity effects according to Kada and coworkers (Morita *et al*., 1978; Kada, 1983).

MMS is a monofunctional alkylating agent that interacts mainly with N-7 guanosine or N-3 adenosine and induces DNA lesions after the treatment. The most DNA lesions detected have the character of ALS, and true SSB represented only a minor fraction (Dipple, 1995; Horvathova *et al*., 1998). These DNA lesions are mainly processed via base excision repair (BER), a mechanism that is responsible for the removal of several classes of damage from DNA employing in early step-specific glycosylases, each recognizing and removing a subset of base damage. *N*-alkylpurines are removed from DNA by BER via *N*-alkylpurine-DNA glycosylases (APDG) (Lindahl *et al*., 1997). CSE and crocin may affect the MMS-induced genotoxicity by enhancing BER through increasing the APDG activity or expression.

Taking together, the findings of the present study and other previous observations indicate that saffron extract and its crocin constituents can inhibit the genotoxicity/carcinogenicity of chemicals with various mechanisms of

action, which should be investigated further using different *in vitro* system assays and different experimental designs.

In conclusion, the results of the present study showed that aqueous extract of CSE and crocin clearly repressed the genotoxic potency of MMS, as measured by the comet assay, in different mice organs.

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