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Study on Genotoxicity of Crocin, a Component of Gardenia Fruit, in Bacterial and Mammalian Cell Systems

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Abstract

Crocin is one of the major components of gardenia fruit and saffron which are widely used as natural food colorants and as traditional Chinese medicines. However, the genotoxicity data on crocin are not sufficient for safety evaluation. The purpose of this study was the examination of the genotoxicity on crocin from gardenia yellow in bacterial and mammalian cells, using various genotoxic battery testing assays and the influence of crocin on methyl methanesulfonate (MMS) and H_2O_2-induced DNA damage *in vitro*, using single cell gel electrophoresis (comet) assay. From results, no considerable mutagenicity and clastogenicity were seen in bacteria and mammalian cells treated with crocin, by Ames test, chromosomal aberration assay, $tk^{+/-}$ gene forward mutation assay and comet assay. And, post-treatment with crocin significantly suppressed H_2O_2-induced DNA damage in a dose-dependent manner. In conclusion, the findings of the present study and other previous observations indicate that crocin has no genotoxic potential. And it showed that crocin clearly repressed the genotoxic potency of H_2O_2. These results suggest that anti-oxidative effects of crocin may be involved in the protective effects of DNA damage.

Keywords: Crocin, Mutagenicity, Clastogenicity, Genotoxicity, Anti-oxidative

Gardenia fruit (*Gardenia jasminoides* E.) is widely used as a natural food colorant and as a traditional Chinese medicine for treatment of hepatic and inflammatory diseases. "Gardenia yellow" is extracted by ethanol from gardenia fruit. And saffron (*Crocus sativus* L.) is a well-known spice and food colorant commonly consumed in different parts of the world. Saffron is known to have the biological and medicinal properties, as an antispasmodic, diaphoretic, carminative, emmenagogic, sedative and so on[1,2]. And, there is no toxicity when taken in the recommended dosage. During the past few years the antitumoural properties of crude saffron stigma extracts, both *in vitro* and *in vivo*, have been demonstrated[3-5].

Crocin, the digentiobiosyl ester of crocetin, is the main pigment and the main biologically active metabolites of gardenia fruit and dried stigmas of saffron. Crocin is one of the red-colored water-soluble carotenoids found in nature; therefore, it might find numerous applications as a food colorant or antioxidant[1,6,7]. And crocin is known to exert protective effects against acute hepatic damage induced by aflatoxin B1 and dimethylnitrosamine[8] and ROS-induced hepatotoxicity and genotoxicity[9] and to have anti-tumor effects on cellular DNA and RNA synthesis[10,11] and the inhibitory effect on free radical chain reactions by antioxidant properties of crocin[12]. Recently, considerable attention has been focused on the safety and protective effects of gardenia fruit and saffron including crocin because of biological and medicinal properties mentioned above.

In this study, we examined the genotoxicity of crocin from gardenia yellow in bacterial and mammalian cells, using various genotoxic battery testing assays and the influence of crocin on methyl methanesulfonate (MMS) and H_2O_2-induced DNA damage *in vitro*, using single cell gel electrophoresis (comet) assay.

Assessment of Mutation Induction by Crocin in the Bacterial System using Ames Reverse Mutation Assay

The mutagenicity of crocin was assessed in *Salmonella typhimurium* (TA98 and TA100), in the presence and in the absence of metabolic activation system (S9 mixture) by a preincubation method[13]. In

observation of the background lawns of treated bacteria, crocin was cytotoxic at doses 5,000 μg/plate in the absence and presence of S9 mixture, and so, we determined this dose as optimal maximum concentrations of crocin for this assay. Positive controls specific to each of the two tester strains resulted in the expected increases in the number of histidine revertants. As shown in Table 1, no significant increases of revertants in TA98 and TA100 strains were shown at all concentrations of crocin used with and without S9.

Table 1. Mutagenicity of crocin in *Salmonella typhimurium* TA 98 and TA100 in the presence and absence of S9 metabolic activation system.

Compound	Dose (μg/plate)	S9 mix	His+revertants/plate (Mean±S.D.)	
			TA98	TA100
DMSO		−	30±1	161±4
Crocin	313	−	29±3	189±16
	625	−	26±2	177±14
	1,250	−	31±6	156±4
	2,500	−	33±4	149±11
	5,000	−	35±3	162±7
2-NF	1.0	−	1,254±10	−
SA	1.0	−	−	1,040±214
DMSO		+	57±2	168±3
Crocin	313	+	52±10	179±4
	625	+	51±10	182±6
	1,250	+	43±9	190±4
	2,500	+	52±1	189±4
	5,000	+	46±6	184±2
2-AA	0.5	+	1,125±480	−
2-AA	1.0	+	−	1,812±352

DMSO: dimethyl sulfoxide, SA: Sodium Azide, 2-NF: 2-Nitrofluorene, 2-AA: 2-Aminoanthracene

Cytotoxicity of Crocin in L5178Y Cells and CHL Cells

Relative survival of Chinese hamster lung (CHL) cells and L5178Y cells following exposure to a range of concentrations of crocin was determined by trypan blue dye exclusion assay. The survival percentage relative to solvent control (DMSO) was determined as a percentage of the number of cells survived after treatment with or without metabolic activation system. The 50% cell growth inhibition concentration (IC_{50}) of crocin was determined as up to 5,000 μg/mL in CHL cells (Figure 1A). Also shown in Figure 1B, IC_{20} in L5178Y cells was 1,680 μg/mL and 3,200 μg/mL in the absence and presence of S9, respectively.

Assessment of Clastogenicity of Crocin in the CHL Cells using Chromosomal Aberration Assay

The clastogenicity of crocin was assessed by its ability to cause chromosomal aberrations in cultured CHL cells. The types and frequencies of chromosomal aberrations seen in crocin-treated and control cultures for 6 hr and 24 hr crocin treatment are listed in Table 2. The concentration for the assay was determined as 5,000, 2,500 and 1,250 μg/mL in the absence and presence S9. Low frequencies of breaks and fragments were shown in crocin-treated cultures and solvent controls, both with and without S9 mixture. No statistically significant increases in the mean percentage of aberrant cells in the types of aberrations noted between crocin-treated and solvent-control were shown both with and without S9 mixture (Table 2). No

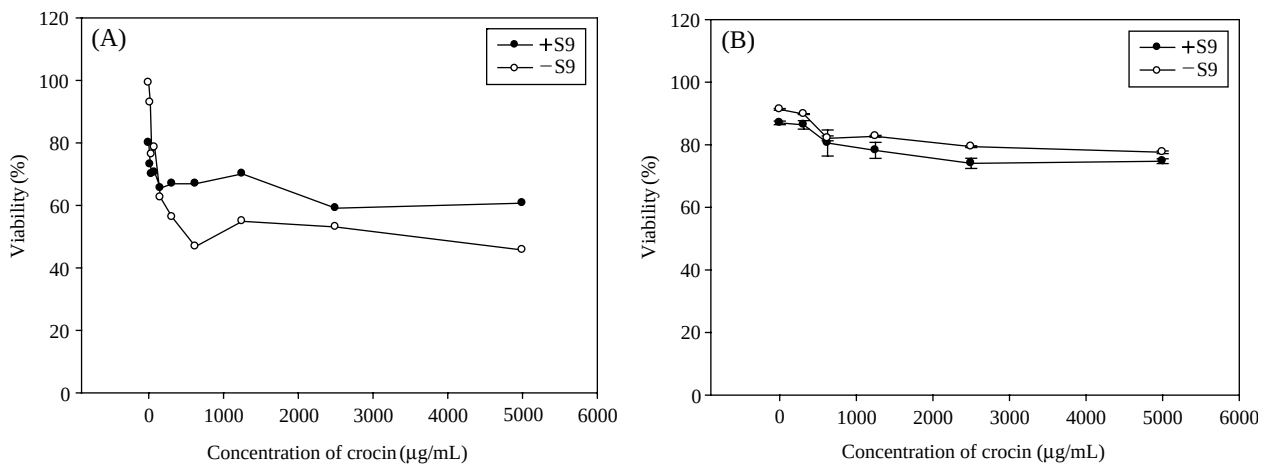

Figure 1. Cytotoxicity of crocin in Chinese hamster lung (CHL) fibroblast cell line (A) and L5178Y mouse lymphoma cell line (B) in the presence and absence of S9 metabolic activation system.

Table 2. Chromosomal aberration assay for crocin in Chinese hamster lung (CHL) cells in the presence and absence of S9 metabolic activation system.

Treatment			S9 mix	Chromosome aberrations/200 cells					Extra aberration				
				Chromatid type		Chromosome type		Total aberration (%)					
Chem	Con.	hr		Br	Ex	Br	Ex		ctg	csg	poly	endo	nor
DMSO	–	6	+	0	0	2	0	1.0	0	0	0	0	198
CP	3.0	6	+	29	35	13	3	40	15	8	0	0	148
	5,000	6	+	2	1	2	0	3.0	4	1	0	0	191
Crocin	2,500	6	+	3	0	1	0	2.0	5	0	0	0	192
	1,250	6	+	1	1	0	0	1.0	3	0	0	0	195
DMSO	–	6	–	2	0	1	0	1.5	4	0	0	0	193
MMC	0.1	6	–	32	31	7	1	35.5	7	3	0	0	139
	5,000	6	–	1	4	1	0	3.0	6	2	0	0	187
Crocin	2,500	6	–	0	1	0	1	1.0	4	0	0	0	196
	1,250	6	–	0	0	1	0	0.5	3	0	0	0	195
DMSO	–	24	–	3	0	1	0	2	3	0	0	0	192
MMC	0.1	24	–	33	30	8	1	36	8	3	0	0	135
	5,000	24	–	3	2	1	1	3.5	7	1	0	0	186
Crocin	2,500	24	–	2	1	0	1	2	5	0	0	0	191
	1,250	24	–	0	1	1	0	1	2	0	0	0	196

Com.: compound, Con.: concentration, Br: breakage, Ex: exchange, ctg: chromatid gap, csg: chromosome gap, poly: polyploid, endo: endoreduplicate, nor: normal, DMSO: dimethylsulfoxide, MMC: mitomycin C, CP: cyclophosphamide

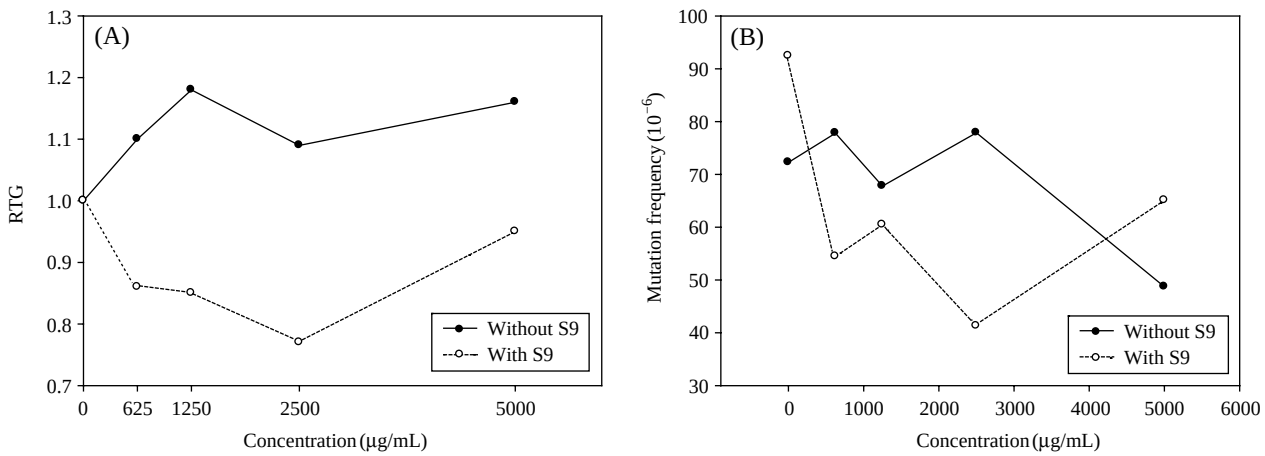

Figure 2. Cytotoxicity (A) and mutagenicity (B) of crocin in L5178Y/$tk^{+/-}$ mouse lymphoma cells. Cytotoxicity (defined as relative total growth) is shown in the left panels and total *tk* mutant frequency is displayed in the right panels. Results are taken from one representative experiment.

unusual types or distributions of aberrations were evident.

Detection of Gross Genetic Alteration on Crocin using L5178Y *thymidine kinase* (*tk*)$^{+/-}$-3.7.2C Mouse Lymphoma Assay (MLA)

The RTG values and the *tk* mutant frequencies (including the small and large colony *tk* mutant frequencies) from one representative experiment with crocin are displayed in Figure 2. The complete data for the assay of crocin are presented in Table 3. No difference was found between the mutant frequency induction by control and crocin with and without S9. A dose response mutagenic effect was not observed at concentrations between 625 and 5,000 μg/mL crocin. Crocin gave mutant frequencies of 77.8, 67.78, 77.88

Table 3. Toxicity and mutagenicity of crocin in L5178Y/$tk^{+/-}$ mouse lymphoma cells.

Treatment (μg/mL)	−S9			Treatment (μg/mL)	+S9		
	% RS	RTG	Mutation frequency ($\times 10^{-6}$)		% RS	RTG	Mutation frequency ($\times 10^{-6}$)
0	100.00	1.00	72.28	0	100.00	1.00	92.42
625	89.98	1.10	77.80 NS	625	124.21	0.86	54.44 NS
1,250	101.56	1.18	67.78 NS	1,250	116.46	0.85	60.46 NS
2,500	96.98	1.09	77.88 NS	2,500	89.01	0.77	41.30 NS
5,000	126.00	1.16	48.72 NS	5,000	109.44	0.95	65.07 NS
Leaner trend			NS	Leaner trend			NS
MMS				CP			
10	96.98	0.74	627.11*	3	100.00	0.77	483.23*

NS: Not significant, *: Statistically significant, $P < 0.05$

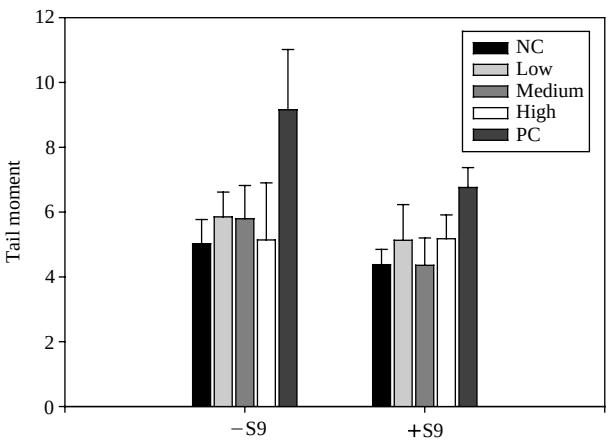

Figure 3. Tail moment of crocin in L5178Y mouse lymphoma cell line assessed by the comet assay. L5178Y cells were treated with indicated concentrations of crocin in the absence and presence S9 metabolic activation system. Values are mean ± S.D. (n=4). Positive controls were MMS (150 μM) in the absence and BaP (50 μM) in the presence of S9 metabolic activation system, respectively. NC: DMSO, Low: +S9 (800 μg/mL), −S9 (420 μg/mL), Medium: +S9 (1,600 μg/mL), −S9 (840 μg/mL), High: +S9 (3,200 μg/mL), −S9 (1,680 μg/mL), PC: +S9 (BaP-50), −S9 (MMS-150).

and 48.72×10^{-6} without S9 and 55.44, 60.46, 41.3 and 65.07×10^{-6} with S9 at each dose of 625, 1,250, 2,500 and 5,000 μg/mL.

Screening of DNA Damage with Crocin using the Single Cell Gel Electrophoresis (Comet) Assay

We also investigated whether crocin could induce subtle DNA damages at IC_{20} concentrations. In this assay, L5178Y cells were treated for 2 hr with 1,680, 840 and 420 μg/mL or 3,200, 1,600 and 800 μg/mL of crocin in the absence or presence of S9, respectively and subjected to the comet assay in optimal conditions as determined by IWGTP. The mean values (± SD) for tail moment by the comet assay are shown in Figure 3. According to the analysis of variance (ANOVA), there were no significant differences between the crocin treated cells and solvent controls in the absence and presence of S9 mixture, suggesting that crocin itself was not induced DNA damages under this experimental condition used in this assay.

Protective Effects of Crocin on MMS and H_2O_2-induced DNA Damage using the Single Cell Gel Electrophoresis (Comet) Assay

L5178Y cells treated 150 μM MMS or 50 μM H_2O_2 as inducing compounds, and then after 30 min, crocin (0, 840, 1,680 μg/mL) was post-treated for 2 hr incubation. And we performed the comet assay (Table 4). Both inducing agents, MMS and H_2O_2 showed significantly higher amount of DNA damage than negative control. Post-treatment of crocin induced dose-dependent decrease of tail moment against H_2O_2 inducing group, however no decreases of tail moment revealed in MMS inducing group.

Discussion

From this study, two major findings were derived about crocin from gardenia yellow. First, no considerable mutagenicity and clastogenicity were shown in bacteria and mammalian cells treated with crocin, as revealed by various genotoxic battery assays. Second, post-treatment with crocin significantly suppressed H_2O_2-induced DNA damage in a dose-dependent manner.

This result is consistent with previous study. It has been reported that saffron or the compounds contains, such as crocin and dimethylcrocin, are not mutagenic

Table 4. Protective effects of crocin on DNA damage induced by H_2O_2 or MMS in L5178Y cells were measured using comet assay.

	Pre-treatment	Concentration of crocin		
		0	840 μg/mL	1,680 μg/mL
Tail moment	No	4.288±4.195	3.640±4.103	3.617±3.548
	50 μM H_2O_2	5.994±5.759	4.549±4.024 *	2.367±2.520*
	150 μM MMS	8.725±7.591	8.345±5.996	8.206±6.383

*Statistically significant, $P < 0.05$

or genotoxic[3-5]. Gardenia yellow was not mutagenic according to the Ames tests[14]. It was reported that gardenia yellow was not clastogenic *in vitro*[15]. However, gardenia yellow was a DNA damage-inducing agent and could increase a number of SCEs significantly[16]. Ozaki *et al*. evaluated on genotoxicity of gardenia yellow and its components, crocetin, gentiobiose (a component of crocin), geniposide and genipin (formed by hydrolysis of gneiposide), without crocin[16]. It indicated clearly that genipin, which is produced from geniposide by human intestinal bacteria[17], was genotoxic. Moreover, they suggested that examination of unidentified components with genotoxic properties in gardenia yellow must be done before using to food as a colorant.

From our results and previous studies, crocin, a component of gardenia yellow and saffron was not mutagenic, clastrogenic and DNA damage inducing in bacterial and mammalian cells with and without S9. Therefore, it suggests that this genotoxic potential of gardenia yellow may be induced by the other component(s) of gardenia yellow, such as not crocin but other iridoid glycosides.

And post-treatment of crocin appeared an attenuated effect on DNA damage against oxidative damage induced by H_2O_2, however, anti-genotoxic effect is not shown against induced by MMS. Crocin scavenges free radicals and thereby may protect cells from oxidative stress[1,11,18-21]. The protective effects of crocin observed in the present study may be related to its antioxidant and radical scavenger properties. It has been shown that antioxidants reduced chemically induced carcinogenesis[22]. Recently, Papandreou *et al.*[23] showed that crocin constituents possess good antioxidant properties, 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[24-28].

In contrast of our results on MMS inducing agent, crocin significantly decreased DNA damage by MMS in different mice organs[27]. Hosseinzadeh *et al*. used pre-treatment method of crocin different with the present study. It suggests that DNA damage already induced by MMS may be not repaired by crocin, with various mechanisms of action, which should be investigated further using different *in vitro* system assays and different experimental designs.

In conclusion, the findings of the present study and other previous observations indicate that crocin has no genotoxic potential. A range of *in vitro* genotoxicity assays was performed with crocin isolated from the Gardenia fruit (Gardenia jasminoides), and was shown to be not mutagenic in bacterial or mammalian cell systems, and not clastogenic in CHL cells. And the results of the present study showed that crocin clearly repressed the genotoxic potency of H_2O_2. These results suggest that anti-oxidative effects of crocin may be involved in the protective effects of DNA damage.

Materials & Methods

Materials

Crocin was purchased from TCI, Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan). Stock solution of crocin was prepared freshly in dimethylsulfoxide (DMSO) before use. Eagles minimum essential medium (EMEM), RPMI-1640, 0.25% trypsin-EDTA, trypan blue, colcemid, fetal bovine serum (FBS) and horse serum were the products of Gibco BRL Life Tech. Inc. (Gaithersburg, USA). Low melting agarose was a product of Amresco (Solon, OH, USA). Methylmethanesulfonate (MMS, CAS no. 66-27-3) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Trifluorothymidine (TFT), cyclophosphamide and sodium bicarbonate were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of analytical grade or the highest grade available. The preparation of rat liver S9 fraction for metabolic activation system was previously reported[14,29]. The S9 fraction prepared was stored immediately at −80°C before use.

Cell Lines and Culture

A clonal sub-line of a Chinese hamster lung (CHL) fibroblast cells was obtained from the National Institute of Health Sciences, Tokyo, Japan. The karyotype of CHL cells consisted of 25 chromosomes. The cells had been maintained by 3-4 day passages and grown in a monolayer with EMEM supplemented with 10% FBS, 50 units/mL penicillin and 50 μg/mL streptomycin. The mouse lymphoma L5178Y cell line (*tk*$^{+/-}$-3.7.2c) was cultivated in 90% RPMI-1640 (Life Technologies, MD, USA) with 1 mM sodium pyruvate, 0.1% pluronic supplemented with 10% heat-inactivated horse serum and antibiotics. These cells were maintained at 37°C in humidified 5% CO_2 atmosphere.

Ames *Salmonella* Bacterial Mutagenicity Assay

The dose range for test chemical was determined by performing a toxicity assay using strain *Salmonella typhimurium* TA 100 and half-log dose intervals of the test substance up to 5 mg/plate. The mutagenicity assay was performed by mixing one of the tester strains which was cultured overnight, with the test substance in the presence and in the absence of S9 mixture condition. Then, incubating the mixture in water bath for 30 min at 37°C and after incubation, the mixture mixed with top agar containing a minimal amount of histidine and then poured onto the surface of a γ-ray sterile Petri dish (Falcon, USA) containing 25 mL of solidified bottom agar. The finished plates were incubated for 48 hr at 37°C, and revertant colonies were counted later. Negative control plates containing no added test chemical but positive control plates containing appropriate amounts of chemicals known to be active were included with each tester strain (Table 1). All platings were done in triplicate, and the results were tabulated as the mean ± standard deviation for each condition. A response was considered to be positive in our criteria if there was a dose-dependent increase in revertants per plate resulting in at least a doubling of the background reversion rate for strains TA 98 or TA 100.

Cytotoxicity (Cell Growth Inhibition)

Cytotoxicity of crocin was checked by the trypan blue exclusion assay. For the determination of cytotoxicity, 1×10^5 CHL cells or 1×10^6 L5178Y cells were treated to various concentrations of crocin in 12 well plate in the absence and presence S9 metabolic activation system for 6 hr or 2 hr, respectively. Cell viability of treated chemical was related to controls that were treated with the solvent. All experiments were repeated twice in an independent experiment.

In vitro Chromosomal Aberrations Assay in CHL Cells

The clastogenicity of crocin was evaluated for its ability to induce chromosomal aberrations in CHL cells. Concentration selection for this assay was based on solubility (testing was performed up to precipitating concentrations, 5 mg/mL, whichever was lower), and determination of cytotoxicity. In the absence and in the presence of S9 mixture, cultures were treated for 6 hr with the crocin of three different doses, including the IC_{50} value as a maximum dose, and then maintained for 18 hr in the fresh medium to adjust a time equivalent to about 1.5 normal cell cycle lengths. Cyclophosphamide (CP) and mitomycin C (MMC) were used as a positive control in combination with or without S9 mixture, respectively. After 22 hr incubation, 2 hr further incubated in the presence of colcemid, metaphase cells were harvested by trypsinization and centrifugation. The cells were swollen by adding with hypotonic (0.075 M) KCl solution for 20 min at 37°C, and washed three times in ice-cold fixative (methanol : glacial acetic acid=3 : 1, A few drop of cell pellet suspension were dropped onto pre-cleaned glass microscope slides, and-dried in the air. Slides were stained with 5% Giemsa buffered solution. The number of cells with chromosomal aberrations was recorded on 200 well-spread metaphase. The classification of aberration types referred to JEMS-MMS[30]. Aberration frequencies, defined as aberrations observed divided by number of cells counted, were analyzed using Fishers exact test[31] with Dunnetts adjustment and compared with results from the solvent controls.

L5178Y *tk*$^{+/-}$-3.7.2C Mouse Lymphoma Assay (MLA)

To prepare working stocks for gene mutation experiments, cultures were purged of *tk*$^{+/-}$ mutants. Each experiment started with working stock. For treatment, cells were centrifuged and suspended at a concentration of 0.5×10^6 cells in 10 mL of medium in 15 mL polystyrene tubes. Crocin at each concentration were added and these tubes were gassed with 5% CO_2 in air and sealed with and without S9 mixture. The cell culture tubes were placed on a roller drum and incubated at 37°C for 3 hr. At the end of the treatment period, the cell cultures were centrifuged and washed. We conducted preliminary experiments to determine the solubility and cytotoxicity of the test chemicals. Cytotoxicity was determined by relative survival (RS) and relative total growth (RTG) following 3 hr treatments at concentrations up to 5 mg/mL, usually regardless of solubility. The recommended highest concentration was one with a 10-20% RS

and/or RTG. Mutant selection was performed using the modified microwell version of the assay as described by Clements *et al.*[32]. Simply, the treated cells in medium containing 3 μg TFT/mL for selection or without TFT for cloning efficiency were distributed at 200 μL/well into 96-well flat-bottom microtiter plates. For mutant selection, two plates were seeded with ~2,000 cells/well. For cloning efficiency, two plates were seeded with ~1 cell/well. All plates were incubated in 5% CO_2 in air in a humidified incubator at 37˚C. After 11-13 days incubation, clones were counted and the colony size distribution was determined. Mutant frequencies were calculated using a statistical package (Mutant™; UKEMS, York, UK) in accordance with the UKEMS guidelines.

Single Cell Gel Electrophoresis (Comet) Assay

DNA damages were detected by the alkaline version of standard comet assay described by Singh *et al.*[33,34] with minor modifications[35,36]. For evaluation of genotoxicity of crocin, 8×10^5 cells were seeded into 12 wells plate (Falcon 3043) and then treated with crocin. At all doses of crocin used in the experiment, the cell viability exceeded 80%. In the experiments, parallel cultures were performed and benzo[a] pyrene (BaP) and methyl methanesulfonate (MMS) were used as a positive control in the presence or absence of S9 mixture, respectively. For evaluation of the protective effects of crocin on DNA damage induced by MMS and H_2O_2, L5178Y cells were treated to 150 μM MMS or 50 μM H_2O_2 for 30 min and then, crocin of each concentration was co-treated without S9. After treatment with crocin for 2 hr, cells were centrifuged for 3 min at × 100 g (about 1,200 rpm), and gently resuspended with PBS and 100 of the cell suspension was immediately used for the test. Cells were mixed with 0.1 mL of 1% low melting point agarose (LMPA) and added to fully frosted slide (Fisher Scientific, PA, USA), had been covered with a bottom layer of 100 μL of 1% normal melting agarose (Amresco, OH, USA). The cell suspension was immediately covered with cover glass and the slides were then kept at 4˚C for 5 min to allow solidification. After removing the cover glass gently, the slides were covered with a third layer of 100 μL of 0.5% LMPA by using a cover glass and then the slides were kept again at 4˚C for 5 min. The cells embedded in the agarose on slides were lysed for 1.5 h in reaction mixture of 2.5 M NaCl, 0.1 M Na_2-EDTA, 10 mM Tris -HCl (pH 10), 1% Triton X-100 at 4˚C. Slides were then placed in 0.3 M NaOH containing 1 mM Na_2-EDTA (approximately pH 13) for 20 min to unwind DNA before electrophoresis. Electrophoresis was conducted at 25 V and approximately 300 mA for 20 min at 4˚C. After the electrophoresis, the slides were stained by 50 μL of ethidium bromide, and then covering the slide with a cover glass. Image of 100 randomly selected cells (50 cells from each of two replicate slides) was analysed each sample. All experiments were repeated in an independent test. Measurement was made by image analysis with Komet 3.1 (Kinetic Imaging Limited, Liverpool, UK) system, determining the mean tail moment (percentage of DNA in the tail times tail length) of the 50 cells. Differences between the control and the other values were tested for significance using one way of analysis of variance (ANOVA).

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