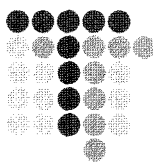

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Genotoxicity Assessment of Gardenia Yellow using Short-term Assays

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Abstract

Gardenia yellow, extracted from gardenia fruit, has been widely used as a coloring agent for foods, and thus, safety of its usage is of prime importance. In the current study, short-term genotoxicity assays were conducted to evaluate the potential genotoxic effects of gardenia yellow. The gardenia yellow used was found to contain 0.057 mg/g of genipin, a known biologically active compound of the gardenia fruit extract. Ames test did not reveal any positive results. No clastogenicity was detected by a chromosomal aberration test, even on evaluation at the highest feasible concentration of gardenia yellow. Gardenia yellow was also shown to be non-genotoxic using an *in vitro* comet assay and a micronucleus test with L5178Y cells, although a marginal increase in DNA damage and micronuclei frequency was reported in the respective assays. Additionally, *in vivo* micronucleus test results clearly demonstrated that oral administration of gardenia yellow did not induce micronuclei formation in the bone marrow cells of male ICR mice. Taken together, our results indicate that gardenia yellow is not mutagenic to bacterial cells, and that it does not cause chromosomal damage in mammalian cells, either *in vitro* or *in vivo*.

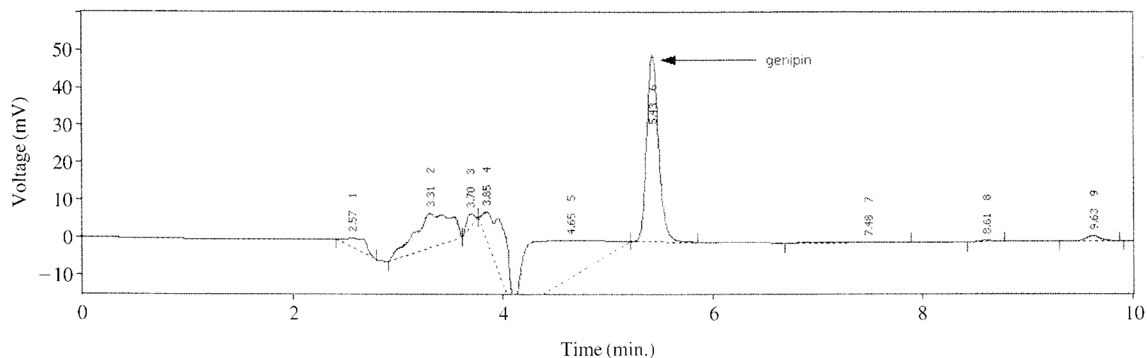
Keywords: Gardenia yellow, Chromosomal aberration test, Micronucleus assay, Comet assay, Genotoxicity, Risk assessment

Gardenia yellow, extracted from gardenia fruit, has been widely used as a natural colorant in food preparations. Gardenia yellow is also a traditional herbal medicine, administered for its antihypertensive and antipyretic effects in East Asian countries, including Korea, Japan, and China. The use of gardenia yellow has been increasing, but the data concerning its toxicity are controversial. Several studies have reported on the toxicity of constituents or metabolites of gardenia yellow; in one such study, a single oral administration of gardenia yellow was found to be hepatotoxic in rats[1]. Conversely, it has also been reported that there were no severe toxic effects associated with ingesting gardenia yellow powder containing 2.783% geniposide over a three-month period[2]. Further, gardenia yellow did not induce carcinogenic activity in mice (http://potency.berkeley.edu/chempages/GARDENIA%20YELLOW.html), and was found to be non-genotoxic using Ames test[3]. Other available genotoxicity data are insufficient for assessing the risks associated with ingesting gardenia yellow.

The major constituents of Gardenia fruit are iridoid glycosides, including geniposide, gardenoside, gardoside, shanzhiside, scandoside methyl ester, methyl deacetyl asperulosidate geniposidic acid, 1-*O*-acetylgeniposide, and genipin-1-β-gentiobioside[4,5]. One of these, geniposide, is recognized as having choleretic effects[6]. Especially, the hepatotoxic effect ascribed to gardenia yellow has been attributed primarily to its geniposide content[7]. However, it has been reported that the effects of geniposide are due to genipin, formed via the hydrolysis of geniposide by intestinal bacteria[8,9]. Genipin has been found to exert several biological activities, including genotoxicity[3,10]. Crocin pigment, another main component of gardenia yellow, has been found to have no genotoxic effects in either bacterial or mammalian systems[11].

Short-term genotoxicity testing relies on the premise that the induction of genetic damage increases the incidence of genetic diseases, such as cancer, and is especially useful as a primary screening technique for carcinogens. In the current study, the whole genotoxic profile of gardenia yellow used in Korea was investigated. Five assessment methods were used as follows: the Ames test, an *in vitro* chromosomal aberration test

(A) Standard compound (genipin)



(B) Gardenia yellow sample

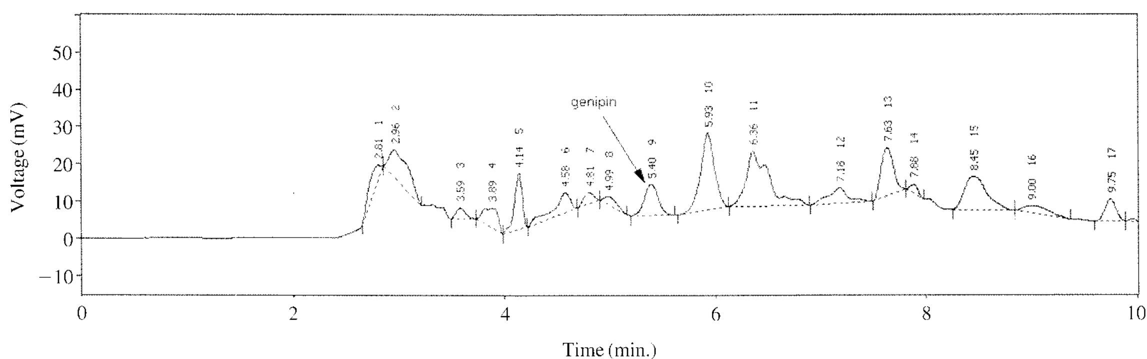


Figure 1. HPLC Chromatogram of Standard Compounds (A) and gardenia yellow sample (B) at 240 nm. The peak observed at RT 5.40 min was confirmed as a genipin by the spike test.

using CHL cells, an *in vitro* comet assay (a single-cell gel electrophoresis assay) using L5178Y cells, an *in vitro* micronucleus test using L5178Y cells, and a micronucleus test using ICR mice. The genipin content of gardenia yellow preparations was determined by HPLC analysis.

The Determination of Genipin Content in Gardenia Yellow

Because genipin has been reported to be genotoxic[3], we determined the genipin content of the gardenia yellow batch used in the current study. This was done by HPLC analysis, using the method described by Ueno *et al.*[12], with some modifications. Figure 1 shows the HPLC profiles of a genipin standard and a gardenia yellow sample recorded at 240 nm. The genipin content of gardenia yellow was found to be 0.057 ± 0.003 mg/g (mean $\pm$ SD, n=3).

Mutagenicity Test-Bacterial Reverse Mutation Assay

The mutagenicity of gardenia yellow was evaluated and no toxicity was observed up to a maximum dose of 5,000 μg/plate. The results of an Ames test with gardenia yellow are shown in Figure 2. Compared to a vehicle control, there was no increase in the number of revertant colonies with any dose or strain. There were also no antibacterial effects, such as a decrease in the number of colonies, observed for any strain.

In vitro Clastogenicity Tests

In vitro Chromosomal Aberration Assay

Results of an *in vitro* chromosomal aberration assay are shown in Table 1. For the continuous treatments of CHL cells with doses up to 5,000 μg/mL, the percentage of total aberrations excluding gaps of gardenia yellow was 0.0%. For short-term treatments, the percentage of total aberrations excluding gaps of gardenia yellow was less than 0.5%, with or without using an S9 mix. Thus, gardenia yellow was demonstrated to be non-clastogenic up to the highest feasible concentration that could be evaluated. As expected, there was a significant increase in the number of aberrant metaphases with positive controls, cyclophosphamide (CPA) treatment for 6 h+S, and mitomycin C (MMC) treatment for either 6 h-S or 24 h-S.

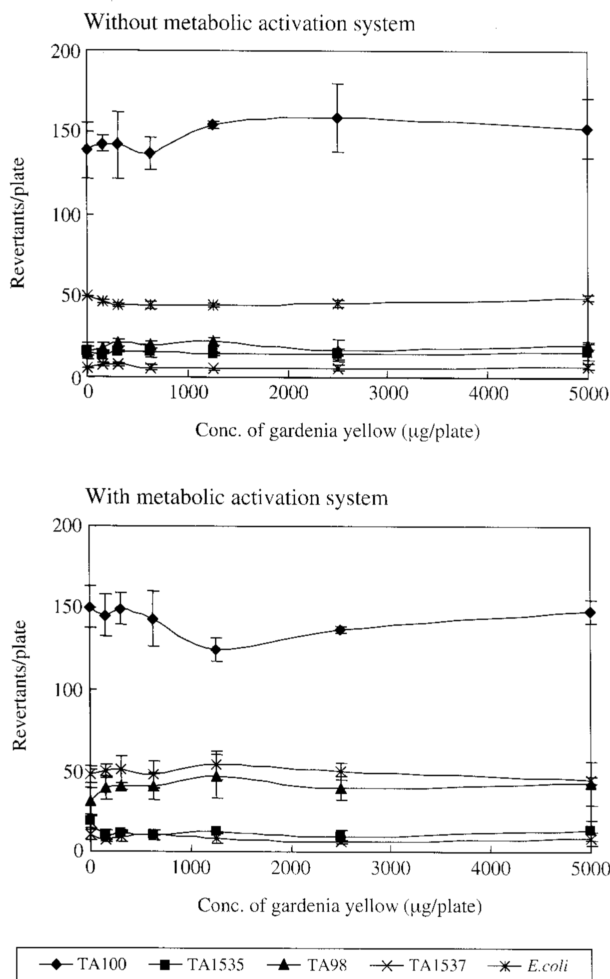


Figure 2. Effect of gardenia yellow on revertant colonies in the Ames assay. Five test strains (TA100, TA1537, TA98, TA1535, *E. coli* WP2 *uvrA*) were exposed to gardenia yellow and incubated for 48 h. Data were expressed as the mean numbers of colonies ± SD from triplicate plates/concentration.

In vitro Micronucleus Assay

Table 2 shows the results of an *in vitro* micronucleus test, performed at the high concentration of 5,000 μg/mL. Cytochalasin B has been previously shown to increase the level of spontaneous micronucleated cells in the L5178Y cell line[13]. Thus, in the current study the *in vitro* micronucleus test was performed without cytochalasin B, which is generally used to ensure that the analyzed cells have completed a division[14]. There was a marginal increase in the number of micronucleated cells, but the difference was not statistically significant. Thus, in concentrations up to 5,000 μg/mL, gardenia yellow tested negative with all treatment schedules. In comparison to a negative control group, the positive controls used in the current study (CPA, MMC, and colchicine) induced statistically significant increases in micronucleated cell numbers.

In vitro Comet Assay

Comet assays were performed using a standard 3 h exposure time with short treatments, in both the absence and presence of an S9 mix, and a 24 h exposure time for continuous treatments with the high concentration of 5,000 μg/mL. Results of an alkaline comet assay with L5178Y $tk^{+/-}$ cells are shown Table 3. Slight, but statistically significant, increases in percentage tail DNA were found on a sporadic basis. However, these marginal increases were not concentration-dependent, and none exceeded a maximal fold increase of 2.0. It can thus be concluded that gardenia yellow tested negative with all treatment schedules. There were significant ($P < 0.01$) increases in both percentage tail moment and Olive tail moment observed for positive controls treated with CPA and MMS.

In vivo Assay-Micronucleus Test

Table 4 shows the incidence of micronucleated polychromatic erythrocytes (MNPCEs) per 2,000 polychromatic erythrocytes (PCEs). Compared to a vehicle control, up to 5,000 mg/kg gardenia yellow did not induce an increase in MNPCEs. The number of MNPCEs found in the current study for vehicle (2.40 ± 1.95) and positive controls (23.20 ± 4.02, $P < 0.05$) are within the expected range for ICR mice. In addition, gardenia yellow had no remarkable effects on the mean number of PCEs per 200 erythrocytes, a parameter used to assess cytotoxicity.

Discussion

Food-grade gardenia yellow, used as a natural food colorant, was previously found to be non-mutagenic using Ames test[3,15]. However, there are few data concerning the potential clastogenicity of gardenia yellow. There is one report of gardenia yellow inducing a significant dose-dependent increase of sister chromatid exchange frequency[3]. Thus, it is of interest to determine whether gardenia yellow, used as a food additive in Korea, contains detectable levels of cell mutagens. Consistent with previous work[3], the Ames test results of the current study showed that gardenia yellow was not mutagenic, irrespective of metabolic activation with S9. The potential clastogenic effects of gardenia yellow in mammalian cells were investigated using three short-term *in vitro* genotoxicity tests: A chromosomal aberration assay, a micronucleus test, and an alkaline comet assay. There was little clastogenicity seen with the *in vitro* chromosomal aberration assay. Further, despite a marginal increase in the number of micronucleated cells, gardenia yellow was found

Table 1. *In vitro* chromosomal aberration assay for gardenia yellow.

Conc. (μg/mL)	S9 mix	Times[a)] (hours)	Aberrant metaphases excluding gaps (%)	Aberrant metaphases including gaps (%)
6 h treatment				
0	+	6-18	0.0[b)]	0.5
1250	+	6-18	0.0	4.0
2500	+	6-18	0.0	1.5
5000	+	6-18	0.0	1.5
CPA 5	+	6-18	34.5**[c)]	42.5
6 h treatment				
0	−	6-18	0.5	1.0
1250	−	6-18	0.0	2.0
2500	−	6-18	0.0	1.5
5000	−	6-18	0.5	2.5
MMC 0.1	−	6-18	11.5**	15.0
24 h treatment				
0	−	24-0	0.0	2.0
1250	−	24-0	0.0	1.0
2500	−	24-0	0.0	1.0
5000	−	24-0	0.0	1.5
MMC 0.1	−	24-0	24.5**	28.5

[a)]Time, Chemical treatment time-recovery time. [b)]Means of duplicate cultures; 100 metaphases were examined per culture. [c)]Fisher's exact test; ** significantly different from the control at $P < 0.01$
Abbreviation: CPA, cyclophosphamide monohydrate; MMC, mitomycin C

Table 2. *In vitro* micronucleus assay for gardenia yellow.

Conc. (μg/mL)	S9 mix	Times[a)] (hours)	Mean micronuclei /1,000 cells[b)]	Relative cell count (%)[c)]
3 h treatment				
0	+	3-21	18.5	100
1250	+	3-21	33.5	95
2500	+	3-21	37.0	96
5000	+	3-21	30.5	95
CPA (5 μg/mL)	+	3-21	64.5**[d)]	64
3 h treatment				
0	−	3-21	21.0	100
1250	−	3-21	31.0	91
2500	−	3-21	31.5	91
5000	−	3-21	31.5	89
MMC (0.125 μg/mL)	−	3-21	148.0**	80
COL (0.2 μg/mL)	−	3-21	58.5**	54
24 h treatment				
0	−	24-0	18.5	100
1250	−	24-0	26.0	116
2500	−	24-0	31.0	123
5000	−	24-0	30.5	117
MMC (0.0625 μg/mL)	−	24-0	155.0**	89
COL (0.2 μg/mL)	−	24-0	117.0**	40

[a)]Time, Chemical treatment time-recovery time. [b)]2,000 cells were examined per culture. [c)]RCC=(Cell counts of treated flask/Cell counts of untreated flask) × 100. [d)]Fisher's exact test; $^{**}P < 0.01$
Abbreviation: RCC, Relative cell count; CPA, cyclophosphamide; MMC, mitomycin C; COL, colchicine

to test negative in all treatment schedules of the *in vitro* micronucleus test. Percentage tail DNA data from the alkaline comet assay, which detects primary DNA single- and double-strand breaks and alkali-labile sites[16-19], were analyzed with nonparametric Kruskal-Wallis tests; this approach was chosen in the ab-

Table 3. *In vitro* comet assay for gardenia yellow.

Conc. (μg/mL)	S9 mix	% Tail DNA[a]	Olive tail moment	Relative cell count (%)[b]
3 h treatment				
0	+	7.86±3.91	5.51±3.23	100
1250	+	9.81±5.64*	8.95±9.77	84.4
2500	+	10.05±5.23**	6.75±3.46	82.0
5000	+	10.51±5.11**	8.30±5.32	92.6
3 h treatment				
0	−	7.41±4.55	5.14±5.15	100
1250	−	9.59±6.09*	5.73±3.73	97.9
2500	−	9.69±5.25*	7.44±4.56	108.2
5000	−	9.63±5.56*	8.76±7.34	93.1
24 h treatment				
0	−	6.46±3.85	4.41±3.50	100
1250	−	11.65±7.18**	8.28±5.62	101.8
2500	−	11.00±6.89**	7.75±4.73	83.0
5000	−	10.00±5.42**	8.08±7.09	97.7
Positive controls				
CPA (10 μg/mL, 3 h)	+	19.29±12.54**	11.51±6.96	54.8
H_2O_2 (200 μM, 30 min)	−	88.59±5.17**	78.60±15.37	71.1
MMS (0.1 mM, 3 h)	−	77.78±6.15**	48.54±6.15	74.0

*$P<0.05$, statistically significant vs. negative control according to Kruskal-Wallis test with post-test.
[a]100 cells were examined per culture. [b]Viability measured by trypan blue exclusion; expressed as percentage of absorbance of control

Table 4. *In vivo* micronucleus assay for gardenia yellow.

Dose (mg/kg)	No. of animal	MNPCE/2,000 PCEs (Mean±S.D.)	PCE/(PCE+NCE) (Mean±S.D.)
0	5	2.40±1.95	0.47±0.02
1250	5	1.80±1.10	0.43±0.02
2500	5	2.20±1.64	0.44±0.04
5000	5	3.20±1.48	0.46±0.03
MMC (1)	5	23.20±4.02*	0.40±0.05

*Significantly different from the vehicle control at $P<0.05$ (Fisher's exact test)
Abbreviations: PCE, Polychromatic erythrocyte; NCE, Normochromatic erythrocyte; MNPCE, PCE with one or more micronuclei; MMC, mitomycin C (positive control)

sence of a consensus on standard statistical methods for analyzing comet assay data[19]. Gardenia yellow was found to induce slight, but statistically significant, increases in percentage tail DNA. However, this finding was deemed to have no biological relevance, because the effect was not concentration-dependent, and did not exceed a maximal fold increase of 2.0. Many positive *in vitro* test results have proven to be irrelevant for humans; which is because *in vitro* short-term tests for genotoxicity can never fully mimic *in vivo* conditions. It is thus required that assessments of carcinogenic potential include an evaluation of genotoxic damage under *in vivo* conditions. The *in vivo* results of the current study clearly demonstrate that orally administered gardenia yellow did not induce micronuclei formation in the bone marrow cells of male ICR mice. On the basis of erythropoietic kinetics and the recommendations and observations of other research groups[20-22], mice were sacrificed 24 h after the final administration of gardenia yellow. We stained our slides with the permanent stain Giemsa because it does not fade even after exposure to strong light during scoring.

Taken together, the negative results of our five short-term genotoxicity tests suggest that gardenia yellow is safe for use as a natural food additive. Additionally, our microarray analysis on L5178Y cells revealed that gardenia yellow does not significantly alter the expression level of many genes thought to be involved in genotoxic stress (manuscript in preparation). This supports the idea that there is no genotoxicological risk associated with using gardenia yellow as a food supplement. However, Ozaki *et al.*[3] have reported a genotoxicity of genipin, which can be produced from geniposide, the principal constituent of gardenia yellow. The Korean Food Additives Codex already lists specifications concerning the purity of commercial food-grade gardenia yellow (Korean Food Additives Codex, 2008). However, HPLC analysis showed the commercial gardenia yellow samples assessed in the current study as containing 0.057% genipin (0.057± 0.003 mg/g). Therefore, given its potential to influence the safety of gardenia yellow, it will be important to

determine the levels at which genipin is itself safe.

Materials & Methods

Test Article and Chemicals

Gardenia yellow in circulation in Korea was purchased at a local marketplace. Chemical and microorganism inspection on element standard of gardenia yellow was performed by Korea Advanced Food Research Institute (Seoul, Korea) according to Korean Food Additives Code. Gardenia yellow was dissolved in distilled water and serially diluted to the appropriate concentrations immediately before use. Most chemicals including positive controls such as 4-nitroquinoline 1-oxide (NQO) and cyclophosphamide were obtained from Sigma (St. Louis, MO). MEM medium, RPMI1640 medium, fetal bovine serum, and penicillin-streptomycin were purchased from GIBCO-Invitrogen (Carlsbad, CA). S9, which was prepared from male Sprague-Dawley rats induced with Aroclor 1254, was from Molecular Toxicology Inc. (Boone, NC) and cofactor for S9 mix was from Wako Pure Chem. Ind., Ltd. (Japan). Genipin as a reference compound for HPLC analysis was purchased from Sigma-Aldrich (St. Louis, MO). The reagents and solvents for HPLC were obtained from Mallinckrodt Baker, Inc. (Phillipsburg NJ).

Determination of Genipin Contents by HPLC Analysis

Since it has been reported that genipin has the potential to induce the genotoxicity[3], genipin content in gardenia yellow batch used in this study were determined by HLPC analysis according to the method of Ueno *et al.*[12] with some modifications. To gardenia yellow solution (100 mg/5 mL), 15 mL of ethyl acetate was added. After vacuum evaporation, the gardenia yellow dissolved in 5 mL of methanol was filtered using syringe filter (0.45 μm). Chromatographic analyses were carried out on YL 9100 HPLC system equipped with UV/VIS Detector (NLG Analytical Ltd., UK). Separation of genipin was performed on ZORBAK eclips XDB-C18 column (4.6 mm × 250 mm, 5 μm). The mobile phases were gradient system of water and acetonitrile with a flow rate of 0.7 mL/min and running time was 15 min. Twenty microliter of sample was injected into the column. The separation of genipin was monitored using a UV/VIS detector at 240 nm, and peak analysis and assignment were performed using reference compounds. The peak observed at RT 5.40 min was confirmed as a genipin by the spike test.

Bacterial Reverse Mutation Assay

Salmonella typhimurium strains TA98 and TA1537 (detect frame-shift mutagens), and strains TA100, TA1535 and *Escherichia coli* WP2 *uvrA* (detect base-pair substitution mutagens) were used as tester strains. All of the tester strains were purchased from Molecular Toxicology Inc. (Boone, NC). The mutation assay was performed according to the method of Maron and Ames[23]. A 0.1 mL aliquot of gardenia yellow containing 156.3-5,000 μg per plate, 0.5 mL of S9 mix (or sodium-phosphate buffer, pH 7.4 for S9 negative group), and 0.1 mL inoculum of the tester strain were added to each tube containing 2 mL of top agar. The mixtures were poured onto the Vogel-Bonner minimal agar plates. Plates were incubated at 37°C for 48 h. Triplicate plates were run for each assay.

In vitro Chromosomal Aberration Assay

In vitro chromosomal aberration assay was performed using Chinese hamster lung fibroblast cells (CHL) (ATCC #CRL-1935), which were obtained from American Type Culture Collection (ATCC, Manassas, VA), as described by Dean and Danford[24] with minor modifications. The assay was consisted of short-term (6 h) and continuous (24 h) treatments. Approximately 22 hours after the start of the treatment, colcemid was added to each culture at a final concentration of 0.25 μg/mL. The slides of CHL cells were prepared following the hypotonic-methanol-glacial acetic acid-flame drying-Giemsa schedule for metaphase plate analysis. The 200 metaphases (100 metaphases from each duplicate culture) were selected and analyzed for each treatment group under 1,000× magnification using a light microscope. The results were expressed as mean aberrant metaphases excluding gaps per 100 metaphases.

In vitro Micronucleus Test

The micronucleus assay was performed according to Kirsch-Volders *et al.*[16] with modifications[17]. The L5178Y $tk^{+/-}$ cell line (ATCC #CRL-9518, subclone 3.7.2-C) used in this study was provided by ATCC. The day before treatment, L5178Y mouse lymphoma cells were seeded at 2×10^5 cells/mL. Cells were treated with gardenia yellow for 3 h and harvested after a 21 h recovery period, or treated for 24 h and harvested immediately. The cellular suspension was centrifuged at 1,000 rpm for 5 min and cells were then resuspended in a KCl 0.075 M solution maintained at room temperature for 10 min (mild hypotonic treatment). The methanol/acetic acid (3 : 1)-fixed slides were stained with 10% Giemsa (pH 6.8). Micronuclei were counted in 2,000 cells with well-preserved cytoplasm. Mitomycin C and colchicine were used as direct-acting

positive controls, and cyclophosphamide was used in the presence of S9 as an indirect-acting positive control. The identification of micronuclei was carried out according to Fenech[25].

In vitro Comet Assay

Exponentially growing L5178Y $tk^{+/-}$ cells were seeded at 2×10^5 cells in 12-well plates and cultured for 24 h prior to gardenia yellow treatment, which was carried out for either 3 h or 24 h with the indicated concentrations of 1,259, 2,500 and 5,000 μg/mL. Following gardenia yellow treatment, cells were rinsed twice and resuspended at 2×10^5 cells/mL in ice-cold PBS. The comet assay was performed as described by Kim *et al.*[26] and manufacturer's instruction. Comets were examined at 200X magnification using a fluorescence microscope (excitation filter, 515-560 nm; barrier filter, 590 nm) connected to a CCD camera. Images of 25 randomly selected nuclei per slide (two slides/culture, duplicate/dose) were analyzed using image-analysis software (Komet 5.0, Kinetic Imaging, Liverpool, UK). Tail Intensity (% of tail DNA) was used as the measure of DNA damage. The results are expressed as the mean ± SD (standard deviation). Methyl methanesulfonate was the direct-acting positive control, and cyclophosphamide was the indirect-acting positive control.

In vivo Micronucleus Test

Approximately 6 weeks old specific pathogen free male ICR mice were obtained from Orient Co., Ltd. (Seoul, Korea). The animals were housed in polycarbonate cages. An ambient temperature of 22 ± 3°C, relative humidity of 50 ± 10%, and photoperiod of 12 h was maintained throughout the study. All animals used in this study were cared for in accordance with the principles outlined in the "Guide for the Care and Use of Laboratory Animals", a NIH publication. Gardenia yellow was orally administered two times to groups of six mice at doses of 1,250, 2,500 and 5,000 mg/kg. Mice were killed 24 h after the final administration, and bone marrow was prepared for evaluation with slight modifications of the method reported by Schmid[27]. At least two slides of the cell suspension per animal were made. The air-dried slides were stained with May-Grunwald and Giemsa. Slides were then examined under 1,000X magnification. Small round or oval shaped bodies, size of which ranging about 1/5 to 1/20 of the diameter of polychromatic erythrocyte (PCE), were counted as micronuclei. A total of 2,000 PCEs were scored per animal by the same observer for determining the frequencies of micronucleated polychromatic erythrocytes (MNPCEs). PCE/(PCE+NCE) ratio was calculated by counting 500 cells.

Statistical Analysis

The statistical analyses for *in vitro* chromosomal aberration and *in vitro* micronucleus results were conducted using Statistical Analysis System (SAS) program according to Richardson *et al.*[28]. A significant increase in micronuclei at any one concentration was determined based on a $P < 0.05$ from a one-tailed Fisher's exact test pair-wise comparison of each treatment group to control. A concentration-related response was determined based on a $P < 0.05$ from a one-tailed trend test. In CA test, pair-wise analyses of the percent aberrant cells in treated and control cultures were performed using Fisher's exact test. The result was judged as positive when there was a statistically significant and dose-related increase or a reproducible increase in the frequency of micronucleated cells (MN assay) or aberrant metaphases (*in vitro* CA assay). Statistically significant values that did not exceed the range of historic solvent control values were not considered positive. For statistical analysis of comet assay, the homogeneity of variances of data was tested with Bartlett's test ($P < 0.05$). If the variances of data were not equal, nonparametric Kruskal-Wallis test was used for statistical evaluations ($P < 0.05$ and 0.01).

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