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Genotoxicity Study of Sophoricoside, a Constituent of *Sophora japonica*, in Bacterial and Mammalian Cell System

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ABSTRACT : **Sophoricoside was isolated as the inhibitor of IL-5 bioactivity from *Sophora japonica* (Leguminosae). It has been reported to has an anti-inflammatory effect on rat paw edema model. To develope as an anti-allergic drug, genotoxicity of sophoricoside was investigated in bacterial and mammalian cell system such as Ames bacterial reversion test, chromosomal aberration assay and single cell gel electrophoresis (Comet) assay. As results, in the range of 1,250~40 μg/plate sophoricoside concentrations was not shown significant mutagenic effects in *Salmonella typhimurium* TA 98, TA 100, TA 1535 and TA 1537 strains in Ames test. The 80% cell growth inhibition concentration (IC_{80}) of sophoricoside was determined as above 5,000 μg/ml in Chinese hamster lung (CHL) fibroblast cell and L5178Y mouse lymphoma cell line for the chromosomal aberration and comet assay, respectively. Sophoricoside was not induced chromosomal aberration in CHL fibroblast cell at concentrations of 700, 350 and 175 μg/ml or 600, 300 and 150 μg/ml in the absence or presence of S-9 metabolic activation system, respectively. Also, in the comet assay, the induction of DNA damage was not observed in L5178Y mouse lymphoma cell line both in the absence or presence of S-9 metabolic activation system. From these results, no genotoxic effects of sophoricoside were observed in bacterial and mammalian cell systems used in these experiments.**

Keywords : sophoricoside, bacterial reverse mutation, chromosomal aberration, comet assay

Introduction

The pharmaceutical activity of plants and herbs is well established in popular medicine. However, most of the information about several medicinal herbs does not have any scientific data support, and some of them may have deleterious effects, for example, mutagenic, toxic and carcinogenic activities (Ames, 1983). It has been widely assumed that mutation represents at least one step in carcinogenesis. The evidence supporting this idea is that the majority of mutagens are carcinogens (McCann *et al.*, 1975) and, for at least some compounds, mutagenic potency is closely correlated with carcinogenic potency (Meselson and Russel, 1977). It is, therefore, important to assess the potential mutagenic effect of natural products before they are considered acceptable for medical use (Farnsworth *et al.*, 1985). So, we call attention to the control of such compounds since they may present genetic toxicity and moreover it is very important to check their cancer inducing potentiality by performing traditional and advanced genotoxicity battery including bacterial reversion, *in vitro* DNA break induction in mammalian cells, and *in vitro* chromosomal aberration analysis.

There are ongoing study to identify anti-inflammatory agents from natural resources, especially herbal medicines and wild plants in Korea. As one example of a very poorly studied medicinal plant, sophoricoside ($C_{21}H_{20}O_{10}$, MW = 432.37), one terpenoid constituent was isolated from *Sophora japonica* (Leguminosae) (Min *et al.*, 1999). This is the isoflavone glycoside with a glucose moiety at position 4. Numerous pharmacological effects of flavonoid compounds have been reported, including immunosuppressive and anti-inflammatory effects (Middleton and Kandaswamin, 1994; Fieder *et al.*, 1998). Sophoricoside exhibited potent inhibitory effects on both IL-5, IL-3 and IL-6 bioactivities but was very weak in the inhibitory effect on GM-CSF bioactivity (Yun *et al.*, 2000). Therefore, sophoricoside would induce differential inhibitory effects on interaction of the cytokines to their corresponding receptors. Also, sophoricoside has been reported to have an anti-inflammatory effect on rat paw

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edema models induced by carrageenin or croton oil, and have an antioxidant effect evaluated by *in vitro* and *in situ* liver chemiluminescence (Gabor and Razga, 1991; Fraga *et al.*, 1987).

However, It is very few reports on the toxicity especially genotoxicity of sophoricoside. Therefore, preliminary toxicological investigation of sophoricoside has been performed *in vitro* assay to determine its cytotoxic and mutagenic potentials. In this study, we adopted three methods to assess the genotoxicity of sophoricoside such as Ames bacterial reverse mutation assay (Ames *et al.*, 1973, 1975; Maron and Ames, 1983), chromosomal aberration assay with Chinese hamster lung cells (Ishidate and Odashima, 1977) and single cell gel electrophoresis (comet) assay (Singh *et al.*, 1988, 1994; Ryu *et al.*, 1997) with mouse lymphoma cells.

Materials and Methods

Materials

Sophoricoside was isolated and donated by Dr. Lee from Yeungnam University. Stock solution of sophoricoside 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). All other chemicals used were of analytical grade or the highest grade available. The preparation of rat liver S-9 fraction for metabolic activation system was previously reported (Ames *et al.*, 1973; Maron and Ames, 1983). The S-9 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 days 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

This test performed essentially as described by Ames *et al.* (1973, 1975). Media and positive control chemicals were obtained from commercial sources and were the purest grades available. 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. Strain TA 100 was chosen as the representative tester strain because of its high spontaneous reversion rate. Spontaneous revertant numbers were counted and plotted against the dose of the test chemical to produce a survival curve for the *his*+ genotype.

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 S-9 mixture condition, sodium phosphate buffer added instead of S-9 mixture both in negative and positive control in test tube. 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 *r*-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).

Table 1. Mutagenicity of sophoricoside in *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 in the presence and absence of S-9 metabolic activation system.

Compound	Dose (μg/plate)	S-9 mix	His+revertants/plate (Mean±S.D)			
			TA98	TA100	TA1535	TA1537
DMSO		−	17±2	179±28	12±4	11±2
Sophoricoside	79	−	13±2	191±3	6±1	11±1
	157	−	18±2	180±15	8±3	12±1
	313	−	23±4	172±16	14±2	16±2
	625	−	17±4	169±6	20±2	12±3
	1250	−	18±3	160±35	21±2	15±1
SA	1	−	−	644±32	263±20	−
2-NF	0.2	−	859±6	−	−	−
9-AA	80	−	−	−	−	700±85
DMSO		+	41±18	185±6	12±8	15±5
Sophoricoside	40	+	37±3	194±12	15±4	13±2
	79	+	42±7	168±3	15±2	14±2
	157	+	37±8	177±9	19±5	22±4
	313	+	43±8	160±16	14±7	20±4
	625	+	30±2	164±16	20±2	14±4
2-AA	0.5	+	1125±147	−	−	−
2-AA	1	+	−	1911±185	−	−
2-AA	2	+	−	-	841±26	528±54

DMSO; dimethyl sulfoxide, SA; Sodium Azide, 2-NF; 2-Nitrofluorene, 9-AA; 9-Aminoacridine, 2-AA; 2-Aminoanthracene

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 (1) at least a doubling of the background reversion rate for strains TA 98 or TA 100 and (2) at least a tripling of the background reversion rate for strains TA 1535 or TA 1537.

Cytotoxicity (cell growth inhibition)

Cytotoxicity of cells was checked by the trypan blue exclusion assay. For the determination of cell cytotoxicity, 1×10^5 CHL cells or 1×10^6 L5178Y cells were treated to various concentrations of sophoricoside in 12 well plate in the absence and presence S-9 metabolic activation system for 6 hr or 2 hr, respectively. After the staining of 0.4% trypan blue (Life Technologies, MD, U.S.A), the total number of cells and the number of unstained cells were counted in five of the major sections of a hemocytometer, and then average number of cells per section was calculated. 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 sophoricoside was evaluated for its ability to induce chromosomal aberrations in CHL cells. The experiment was performed as described by OECD (1993) and Ishidate and Odashima (1977) with some minor modifications (Ryu *et al.*, 1993, 1994, 1996a,b, 1998a,b, 2001), which are briefly summarized as follows.

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. Three different doses, including the IC_{50} value as a maximum dose, were prepared and separately added to 3-day-old cultures (approximately 10^5 cells/60 mm dish). In the absence and in the presence of S-9 mixture, cultures were treated for 6 hr with the sophoricoside 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 S-9 mixture, respectively. After 22 hr incubation, the treatment was followed by addition of medium containing colcemid at a concentration of 0.2 μg/ml. Then, 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). After centrifugation, the fixative was removed, and cell pellet suspensions were prepared by pipetting gently. 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 at pH 6.8 for scoring of chromosome aberrations. The number of cells with chromosomal aberrations was recorded on 200 well-spread metaphase cells at the magnification of 1,000 with Axioscope microscope (Karl Zeiss, FRG). The classification of aberration types referred to JEMS-MMS (1988). Breaks less than the width of a chromatid were designated as gaps in our criteria, and not included as chromosomal aberration. The incidence of polyploid and endoreduplicated cells was also recorded when these events were observed. Solvent-treated cells served as controls in this experiment.

CHL cells usually have less than 3.0% cells with spontaneous chromosome aberrations. Aberration frequencies, defined as aberrations observed divided by number of cells counted, were analyzed using Fishers exact test (Altman, 1993) with Dunnetts adjustment and compared with results from the solvent controls. Therefore, data from count up well-spread 200 metaphase cells were expressed as percentages, and then dose-dependent responses and the statistical significance in p-value will be considered as positive results.

Single cell gel electrophoresis (Comet) assay

DNA damages were detected by the alkaline version of standard comet assay described by Singh *et al.* (1988, 1994) with minor modifications (Fairbairn *et al.*, 1995; Ryu *et al.*, 1997; Tice *et al.*, 2000).

For the comet assay, 8×10^5 cells were seeded into 12 wells plate (Falcon 3043) and then treated with sophoricoside. At all doses of sophoricoside 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 S-9 mixture, respectively. After treatment with sophoricoside for 2 hr, cells were centrifuged for 3 min at ×100 g (about 1,200 rpm), and gently resuspended with PBS and 100 μl of the cell suspension was immediately used for the test. Cells were mixed with 0.1 ml of 1% low melting point agarose (LMPA, Gibco BRL, Life Technologies, Inc., MD, USA) and added to fully frosted slide (Cat. No., 12-544-5, 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 slide were kept again at 4°C for 5 min.

The cells embedded in the agarose on slides were lysed for 1.5 hr 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 (about 1 V/cm across the gels) and approximately 300 mA for 20 min at 4°C. All of the steps described above were conducted under yellow light or in the dark to prevent additional DNA damage.

After the electrophoresis, the slides were washed gently to remove alkali and detergents that would interfere with ethidium bromide staining, by placing the slides vertically in glass jar containing 0.4 M Tris (pH 7.5) three times for 10 min. The slides were stained by 50 μl of ethidium bromide in distilled water solution on each slide, 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).

Results and Discussion

Ames reverse mutation assay

The mutagenic potential of sophoricoside was investigated in the S. typhimurium microsomal activation assay. This assay detects materials that cause specific point mutations such as base-pair substitution and frameshift mutation in a bacterial model. The genotoxic evaluations were performed with sophoricoside, in different *S. typhimurium* strains (TA98, TA100, TA1535 and TA1537), in the presence and in the absence of S-9 mixture. Positive controls specific to each of the four tester strains resulted in the expected increases in the number of histidine revertants. In observation of the background lawns of treated bacteria, sophoricoside was cytotoxic at doses above 1,250 μg/plate and 625 μg/plate in the absence and presence of S-9 mixture, respectively, and so, we determined as optimal maximum concentrations of sophoricoside for this assay. As shown in Table 1, no significant increase of revertants in four cell lines at all concentrations of sophoricoside used. These results suggest that sophoricoside is not mutagenic in *S. typhimurium* TA98, TA100, TA1535 and TA1537 strains both in the presence and absence of metabolic activation in this assay.

Cytotoxicity of sophoricoside

Relative survival of CHL cells and L5178Y cells following exposure to a range of concentrations of sophoricoside 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

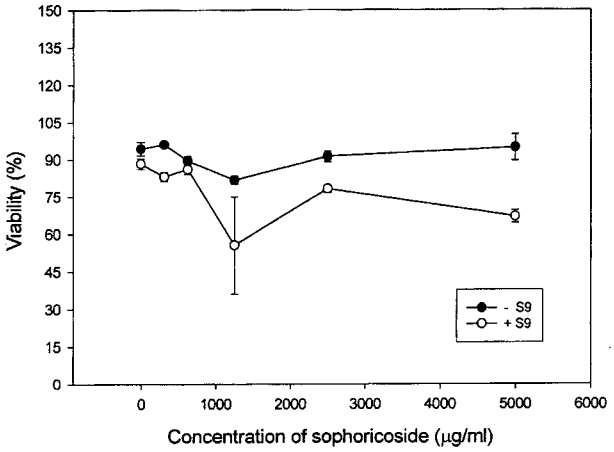

Fig. 1. Cytotoxicity of sophoricoside in Chinese hamster lung (CHL) fibroblast cell line in the presence and absence of S-9 metabolic activation system.

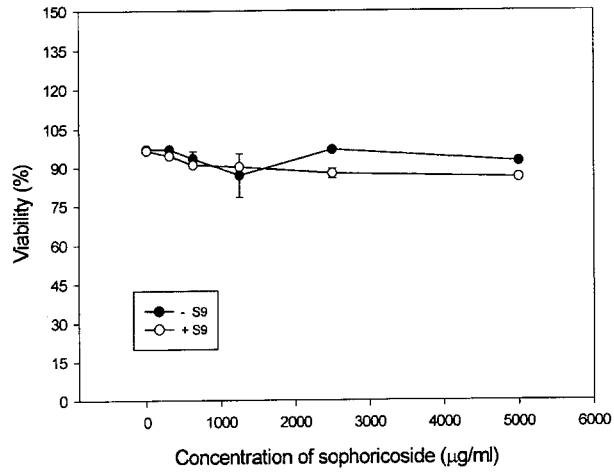

Fig. 2. Cytotoxicity of sophoricoside in L5178Y mouse lymphoma cell line in the presence and absence of S-9 metabolic activation system.

Table 2. Chromosomal aberration assay for sophoricoside in Chinese hamster lung (CHL) cells in the presence and absence of S-9 metabolic activation system.

Treatment				Chromosome aberrations / 200 cells					Extra aberration				
				Chromatid Type		Chromosome Type		Total Aberration (%)					
Compound	Con. (μg/ml)	h	S-9 mix	Br	Er	Br	Ex		ctg	csg	poly	endo	nor
DMSO	–	6	+	2	0	0	0	0.5	3	0	0	0	195
CP	3.0	6	+	29	35	13	3	40	15	8	0	0	148
Sophoricoside	600	6	+	2	1	1	0	2.0	4	1	0	0	191
	300	6	+	2	1	1	0	2.0	5	0	0	0	92
	150	6	+	1	0	0	0	0.5	6	0	0	0	193
DMSO	–	6	–	1	1	0	0	1.0	3	0	0	0	195
MMC	0.3	6	–	32	31	7	1	35.5	7	3	0	0	139
Sophoricoside	700	6	–	3	1	0	0	2.0	5	1	0	0	190
	350	6	–	2	0	0	0	1.0	4	1	0	0	193
	175	6	–	1	0	0	0	0.5	3	1	0	0	195

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.

sophoricoside was determined as up to 5,000 μg/ml in CHL fibroblast cells (Fig. 1). Also shown in Fig. 2, exposure of 5,000 μg/ml sophoricoside for 2 hr resulted in relative survival exceeded 80% compared to solvent control in mouse lymphoma L5178Y cells.

In vitro chromosomal aberration assay in the CHL cells

The clastogenicity of sophoricoside was assessed by its ability to cause chromosomal aberrations in cultured CHL cells. The types and frequencies of chromosomal aberrations seen in treated and control cultures for 6 hr sophoricoside treatment are listed in Table 2. Sophoricoside was slightly insoluble resulting in formation of precipitate at doses over 600 μg/ml, and preparation of slides for the assay was difficult. So, concentrations for the assay were determined as 700, 350 and 175 μg/ml in the absence of S-9 and as 600, 300 and 150 μg/ml in the presence of S-9 mixture. The solvent (DMSO)-treated control was revealed only 0-1% of spontaneous chromosomal aberrations in 200 metaphase cells. Cyclophosphamide (3 μg/ml) used as an indirect-acting mutagen that requires metabolic activation and mitomycin (0.3 μg/ml) as a direct-acting mutagen, induced remarkable chromosomal aberrations (about 20-40%) in CHL fibroblasts. Low frequencies of breaks and fragments were seen in treated cultures and solvent controls, both with and without S-9 mixture. No statistically significant increases in the mean percentage of aberrant cells in the types of aberrations noted between treated and solvent-control were seen both with or without S-9 mixture (Table 2). No unusual types or distributions of aberrations were evident. The mean percentage of aberrant cells without S-9 mixture ranged from 0.5 to 2%, compared with a mean solvent control value of 1%. For treatment with S-9 mixture, the mean aberrant cell percentage ranged from 0.5 to 2%, compared with a mean solvent control value of 0.5%. From this result, 6 hr treatment of sophoricoside was not revealed clastogenicity both in the presence and absence of S-9 mixture in this assay.

Single cell gel electrophoresis (comet) assay in L5178Y mouse lymphoma cells

We also investigated whether sophoricoside could induce subtle DNA damages at concentrations resulting in no obvious cytotoxic effects. As one of the mechanisms of carcinogenicity, induction of DNA damage was ascertained by a comet assay, which is widely used for the detection and measurement of DNA strand breaks. Since Ostling and Johanson (1984) introduced microelectrophoretic technique, Singh *et al.* (1988) have modified and improved the microgel electrophoresis technique to evaluate DNA damage in single cells under alkaline conditions. The single cell gel electrophoresis (SCGE, comet, microgel electrophoresis) assay is rapid, sensitive, visual and simple technique to quantify DNA strand breaks in individual cells. The intensity and the length of comet images were expressed in terms of the tail moment. However, some variations could be occurred in procedures, laboratories's conditions and kind of cells used. Hence, to overcome and to harmonize these matters in comet assay, International Workshop on Genotoxicity Test Procedure (IWGTP) was held with several topics including comet assay at Washington D.C. on March, 1999 by Environmental

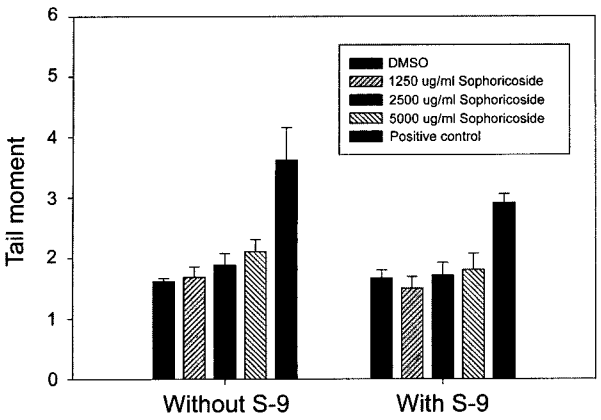

Fig. 3. Tail moment of sophoricoside in L5178Y mouse lymphoma cell line assessed by the comet assay. L5178Y cells were treated with indicated concentrations of sophoricoside in the absence and presence S-9 metabolic activation system. Values are mean±S.D. (n=4). Positive controls were MMS (150 μM) in the absence and BaP (50 μM) in the presence of S-9 metabolic activation system, respectively.

Mutagen Society supported with OECD. Our laboratory (Ryu *et al.*, 1997, 1999, 2001) also involved in this harmonization and published as preliminary form for OECD guideline with Tice *et al.* (2000).

In this assay, L5178Y cells were treated for 2 hr with 5,000, 2,500 and 1,250 μg/ml of sophoricoside in the absence or presence of metabolic activation system, and subjected to the comet assay in optimal conditions as determined by IWGTP. Cell viability at 5,000-1,250 μg/ml ranged from 89 to 98 % (Fig. 2). The results of the comet assay are shown in Fig. 3. The mean values (±SD) for tail moment without and with S-9 mixture were 1.62±0.05 and 1.67±0.13 in the solvent controls, 2.10±0.36 and 1.81±0.27 in the 5,000 μg/ml treated cells, 1.88±0.34 and 1.71±0.21 in the 2,500 μg/ml treated cells, and 1.68±0.23 and 1.49±0.19 in the 1,250 μg/ml treated cells, respectively. According to the analysis of variance (ANOVA), there were no significant differences between the sophoricoside treated cells and solvent controls in the absence and presence of S-9 mixture, suggesting that sophoricoside was not induced DNA damages under this experimental conditions used in this assay.

All three assays were conducted in the presence and absence of S-9 metabolic activation to determine whether the sophoricoside could be converted to mutagenic analogues following bioactivation by microsomal monooxygenase systems. The results of this battery of assays indicate that sophoricoside has no genotoxic potential. In conclusion, a range of in vitro genotoxicity assays was performed with sophoricoside isolated from the sophora japonica plant, and was shown to be not mutagenic in bacterial or mammalian cell systems, and not clastogenic for 6 hr treatment in CHL cells.

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