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Evaluation of the genotoxicity of stevioside and steviol using six *in vitro* and one *in vivo* mutagenicity assays

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Stevioside, a constituent of *Stevia rebaudiana*, is commonly used as a non-caloric sugar substitute in Japan. The genetic toxicities of stevioside and its aglycone, steviol, were examined with seven mutagenicity tests using bacteria (reverse mutation assay, forward mutation assay, *umu* test and *rec* assay), cultured mammalian cells (chromosomal aberration test and gene mutation assay) and mice (micronucleus test). Stevioside was not mutagenic in any of the assays examined. The aglycone, steviol, however, produced dose-related positive responses in some mutagenicity tests, i.e. the forward mutation assay using *Salmonella typhimurium* TM677, the chromosomal aberration test using Chinese hamster lung fibroblast cell line (CHL) and the gene mutation assay using CHL. Metabolic activation systems containing 9000 *g* supernatant fraction (S9) of liver homogenates prepared from polychlorinated biphenyl or phenobarbital plus 5,6-benzoflavone-pretreated rats were required for mutagenesis and clastogenesis. Steviol was weakly positive in the *umu* test using *S.typhimurium* TA1535/pSK1002 either with or without the metabolic activation system. Steviol, even in the presence of the S9 activation system, was negative in other assays, i.e. the reverse mutation assays using *S.typhimurium* TA97, TA98, TA100, TA102, TA104, TA1535, TA1537 and *Escherichia coli* WP2 *uvrA*/pKM101 and the *rec*-assay using *Bacillus subtilis*. Steviol was negative in the mouse micronucleus test. The genotoxic risk of steviol to humans is discussed.

Introduction

Stevioside is a major sweet constituent of the herb, *Stevia rebaudiana* Bertoni, and has been widely used in Japan and Brazil as a non-nutritive sweetening agent (Melis, 1992). The substance has been subjected to various assessments for safety and no serious toxic effects have been reported (Planas, 1968; Akashi and Yokoyama, 1975; Mori *et al.*, 1981; Medon *et al.*, 1982; Hagiwara *et al.*, 1984; Yamada *et al.*, 1985; Aze *et al.*, 1991). Stevioside and crude extracts of *S.rebaudiana* have been determined to be non-mutagenic when tested against some tester strains of *Salmonella typhimurium, Escherichia coli* and *Bacillus subtilis* (Okumura *et al.*, 1978; Fujita *et al.*, 1979; Kawachi *et al.*, 1980). It is known, however, that stevioside is converted to its aglycone steviol by intestinal bacteria when stevioside is orally administered to rats (Wingard *et al.*, 1980; Nakayama *et al.*, 1986). Pezzuto *et al.* (1985) examined the mutagenicity of stevioside and its related compounds and found that steviol showed a dose-related positive response in the forward mutation assay using *S.typhimurium* TM677. Steviol requires metabolic activation for mutagenesis and this reaction seems to be cytochrome P-450-dependent (Pezzuto *et al.*, 1986). To further assess the genotoxicity of stevioside and steviol, we examined their possible mutagenicity, using six *in vitro* and one *in vivo* mutation assays. The results indicated that stevioside is not mutagenic in any of the assays examined but steviol with metabolic activation is mutagenic in the forward mutation assay using *S.typhimurium* TM677, the chromosome aberration test and the gene mutation assay using Chinese hamster lung fibroblast cell line (CHL).

Materials and methods

Chemicals

Stevioside (CAS registry number: 57817-89-7) and steviol (CAS registry number: 471-80-7) were kindly provided by Maruzenn Kasei Ltd, Japan. Stevioside was isolated from the crude extracts of *S.rebaudiana* and steviol was enzymatically prepared from stevioside. The purities of stevioside and steviol were 83.2 and 99.0% respectively, as determined by high performance liquid chromatographic analysis [for stevioside analysis: Lichrosorb NH_2 column, acetonitrile:water (4:1), for steviol analysis: Nucleosil $_5C_{18}$ column, acetonitrile:water (4:1)]. Stevioside and steviol were dissolved in dimethylsulphoxide (DMSO) when used for the mutagenicity tests, unless otherwise indicated. $NADP^+$, NADPH, glucose-6-phosphate (G-6-P), G-6-P dehydrogenase and NADH were purchased from Oriental Yeast Co. Ltd (Tokyo, Japan).

Metabolic activation system

The liver 9000 *g* supernatant fraction (S9) was prepared from Fischer rats (Charles River Japan Co., Chiba, Japan) pretreated with polychlorinated biphenyls (Kanechlor KC-400, 500 mg/kg) 5 days before sacrifice. This S9 was used for all the mutagenicity tests except for *umu* test and the gene mutation assay using CHL cells, in which S9 prepared from Sprague–Dawley rats pretreated with phenobarbital plus 5,6-benzoflavone was used. The latter S9 was purchased from Oriental Yeast Co.

Salmonella/microsome mutagenicity test

The mutagenicity test was carried out according to the method of Maron and Ames (1983) combined with a pre-incubation procedure for 20 min at 37°C. All the *S.typhimurium* tester strains used were provided by Dr B.N.Ames, University of California, Berkeley, CA, USA. Duplicate plates were used for each of five different test substance concentrations.

Forward mutation assay

The assay was carried out with modifications according to the method described by Skopek *et al.* (1978a,b). Briefly, the test sample (50 µl), a log-phase culture of tester strain (800 µl), S9 (100 µl) and cofactor solution (100 µl) made up with G-6-P (5 mg), G-6-P dehydrogenase (2 U), NADPH (5 mg) and $MgCl_2$ (3.3 mg) in minimal medium E (0.5 ml) were added to a small test tube. For the experiments without activation, the test sample (50 µl) was mixed with the log-phase culture (1 ml) alone. After incubation at 37°C for 1 h with shaking (100 r.p.m.), the suspension was centrifuged at 2000 *g* for 15 min and the pellet was resuspended in phosphate-buffered saline

(pH 7.0). To determine the number of 8-azaguanine (8-AG) resistant cells, ~1×10^5 cells were spread on agar plates containing 8-AG (50 μg/ml) To determine toxicity, a diluted suspension was plated on the same plates without 8-AG. After incubation at 37°C for 2 days, the numbers of colonies arising on the mutation and toxicity plates were counted. The mutation frequency was calculated as M×D/S where M is the average number of colonies (mutants) on 8-AG-containing plates, S is the average number of colonies (viables) on plates without 8-AG and D is the dilution factor. The tester strains, *S.typhimurium* TM677 and TM35, were provided by Dr W.G.Thilly, Massachusetts Institute of Technology, Cambridge, MA, USA *Salmonella typhimurium* KH75 was constructed by introducing the plasmid pKM101 into a spontaneous His^+ revertant of *S.typhimurium* TA1975. These strains were cultured overnight using Brain Heart Infusion media and stored at –80°C until use.

umu test

The *umu* test was performed according to the method of Oda *et al.* (1985) with slight modifications. Briefly, the incubation mixture consisted of the test compound dissolved in DMSO (10 μl), a log-phase culture of tester strain *S.typhimurium* TA1535/pSK1002 (240 μl) and either 0.1 M phosphate buffer pH 7.4 (50 μl) or S9 mix (50 μl) containing 5 μl of S9 fraction. The composition of S9 mix was described previously (Oda *et al*, 1985) After incubation for 2 h at 37°C, each mixture was diluted 10 times with TGA medium and further incubated for 3 h at 37°C. After incubation, the cell density (OD_{600}) of each culture was measured and β-galactosidase activity was assayed by the method of Miller (1972). The genotoxic activities of the test substances were defined as specific β-galactosidase activities, and the data are presented as mean values of duplicate or triplicate determinations.

Spore rec-assay

The assay was carried out according to the method of Hirano *et al.* (1982). A paper disk (8 mm in diameter) was placed on the centre of agar plates containing spores of *B.subtilis* either with or without S9. A DMSO solution of the test substance (40 μl) and either buffer solution (20 μl) or cofactor solution (20 μl) were placed on the paper disk. The buffer solution contained 8 μM $MgCl_2$ and 33 μM KCl in 10 mM phosphate buffer pH 7.2. The cofactor solution contained G-6-P and $NADP^+$ (40 and 80 mg/ml of the buffer solution respectively). After incubation at 4°C overnight, the plates were incubated again overnight at 37°C and the diameter of the inhibition zones was measured. *Bacillus subtilis* H17 Rec^+ and M45 Rec^- were provided by the late Dr T.Kada, Department of Induced Mutation, National Institute of Genetics, Mishima, Japan.

Chromosomal aberration test in vitro

A CHL cell line was used (Ishidate and Odashima, 1977). The cells were treated for 24 and 48 h with a test compound at three or more dose levels Stevioside and steviol were dissolved in physiological saline and DMSO respectively. The maximum dose of each test compound was selected from the doses at which >50% cell growth inhibition was observed in a preliminary test. For the metabolic activation, the cells were treated with the S9 mix together with the test compound (Matsuoka *et al.*, 1979). After treatment for 6 h, the reaction mixture was replaced with fresh medium, and then the cells were recultured for 18 h. Both with and without the metabolic activation system, the cells were treated with colcemid (0.2 mg/ml) for 2 h, and chromosome preparations were made using a standard air-dry method. The frequency of the cells with chromosomal aberrations was scored in 100 well-spread metaphases for each dose. Types of structural chromosomal aberrations were classified into five groups: chromatid gaps (ctg) including chromosome gaps (csg), chromatid breaks (ctb), chromatid exchanges (cte), chromosome breaks (csb) and chromosome exchanges (cse) including dicentric and ring chromosomes Polyploid cells were also recorded. The final result of the test compound was judged as follows: negative (–) if the frequency of aberrant cells was <5%, inconclusive (±) if ≥5% but <10%, and positive (+) if ≥10%

Gene mutation assay with cultured mammalian cells

The assay was performed according to the method of Nakayasu *et al.* (1982). CHL cells in logarithmic phase of growth were exposed to steviol dissolved in DMSO for 3 h at 37°C in the presence of S9 mix (1 ml), which contained 4.27 mM G-6-P, 0.75 mM $NADP^+$ and 5% S9 in Eagle's minimum essential medium supplemented with double concentrations of vitamins and amino acids. After the treatment, the cells were washed and collected after trypsinization. For determination of cytotoxicity, the treated cells (200–400 cells) were cultured for 7 days and the numbers of surviving colonies were counted. For determination of mutation frequency, the remaining portion of the cells was cultured in fresh medium for 7 days. After the expression time, an aliquot (2.5×10^5 cells) was transferred to fresh medium containing diphtheria toxin (0.1 Lf/ml), cultured for another 7 days and the number of diphtheria toxin resistant (DT^r) colonies was scored. Another aliquot of the cell suspension (250 cells) was transferred to toxin-free medium to determine the plating efficiency. The mutation frequency was expressed as the number of DT^r cells per 2.5×10^5 survivors, normalized by the plating efficiency.

Micronucleus test

The *in vivo* micronucleus test on steviol was carried out using MS/Ae mice (Aeschbacher *et al*, 1979; Hayashi *et al.*, 1982). Male MS/Ae mice (aged 7 weeks) were purchased from the Hitachi Medical Animals Laboratories (Sanwa, Japan), and were used for the experiments after 2 weeks of acclimation (body weight: 32 8–36.2 g). Mice were given commercial pellets (CE2; Japan Clea) and water *ad libitum* throughout the acclimation and experiments. In each dose group, six mice were used. The test compound suspended in olive oil was administered once by i.p. injection at four dose levels (125, 250, 500 and 1000 mg/kg) Mitomycin C (2.0 mg/kg) was used for the positive control and olive oil for the negative control. All mice were killed by cervical dislocation 24 h after treatment, except that an additional six mice that were injected once with 500 mg/kg of the test compound were killed 48 h after treatment. Femoral marrow cells were flushed out with fetal bovine serum and smeared on clean glass slides Cells were fixed with methanol for 5 min, and stained with 3% Giemsa. The preparations were coded and analysed without any knowledge of the treatment One thousand polychromatic and normochromatic erythrocytes per mouse were scored, and the numbers of micronucleated polychromatic erythrocytes (MNPCEs) and of micronucleated normochromatic erythrocytes (MNNCEs) were recorded. The numbers of micronucleated erythrocytes (MNEs) and the proportion of polychromatic erythrocytes (PCEs) relative to the total erythrocytes were evaluated by observing 1000 erythrocytes on the same slide The results obtained from each dose level were analysed statistically according to the table presented by Kastenbaum and Bowman (1970)

Results

Reverse mutation assays using S.typhimurium and E.coli

Stevioside and steviol were not mutagenic toward *S.typhimurium* TA97, TA98, TA100, TA102 and TA104 either with or without S9 mix at doses up to 5 mg per plate (Table I). They were not toxic to *S.typhimurium* even at the highest dose. Neither stevioside nor steviol was mutagenic in *S.typhimurium* TA1535, TA1537 and *E.coli* WP2 *uvrA*/pKM101 in the presence of S9 mix (data not shown). These results suggest that neither stevioside nor steviol is mutagenic in *S.typhimurium* TA strains and *E.coli* WP2 *uvrA*/pKM101 either with or without metabolic activation.

Forward mutation assay using S.typhimurium TM677

Stevioside induced no significant increase of the mutation frequency of *S.typhimurium* TM677, even at the highest dose of 10 mg/ml, either with or without S9 mix (data not shown). However, steviol induced a significant dose-related increase in the mutation frequency when S9 mix was present (Table II). Steviol increased not only the mutation frequency but also the raw number of 8-AZ resistant colonies (mutants) per plate, ruling out the possibility that the mutagenicity of steviol was an artefact due to the analysis of the data (Procinska *et al.*, 1991). In the absence of S9 mix, steviol did not give rise to an increase in the mutation frequency. To determine the genetic requirements for the mutagenicity of steviol, we compared the sensitivities of three isogenic tester strains in the presence of S9 mix (Figure 1). Of the three strains examined, *S.typhimurium* TM677 (*uvrB, rfa*, pKM101) exhibited much higher sensitivity toward steviol than did *S.typhimurium* TM35 (*uvrB, rfa*) or KH75 (*rfa*, pKM101). These results suggest that steviol is mutagenic to *S.typhimurium* TM677 in the presence of S9 mix and also that *rfa* mutation, deficiency of excision repair and presence of plasmid pKM101 are all required for the maximum mutagenesis.

umu test

Stevioside induced no significant increase in the specific β-galactosidase activity of *S.typhimurium* TA1535/pSK1002

Table I. Results of mutagenicity tests of stevioside and steviol in *S.typhimurium* TA strains

Compound	Solvent	Dose (μg/plate)	S9 mix	Number of revertants per plate				
				TA100	TA98	TA102	TA97	TA104
Stevioside	DMSO	0	–	106	31	295	134	347
		50	–	102	40	320	142	315
		100	–	100	33	319	184	357
		500	–	105	38	314	137	312
		1000	–	117	33	337	172	394
		5000	–	104	61	370	141	487
		0	+	107	34	354	142	572
		50	+	106	50	297	163	557
		100	+	100	46	353	201	606
		500	+	123	51	330	178	574
		1000	+	101	44	367	212	602
Steviol	DMSO	0	–	94	46	364	216	347
		50	–	97	25	437	231	381
		100	–	72	24	387	215	335
		500	–	119	28	398	166	393
		1000	–	121	27	426	182	428
		5000	–	92	NC	NC	NC	233
		0	+	78	21	446	214	572
		50	+	100	20	459	219	550
		100	+	96	22	478	217	585
		500	+	88	17	467	197	583
		1000	+	89	17	425	212	496
		5000	+	122	NC	NC	234	254
Furylfuramide	DMSO	0.02	–	1220				
		0.05	–		590			
Mitomycin C	DMSO	0.01	–			1721		
ICR-191	DMSO	0.1	–				5386	
Methylglyoxal	DMSO	10	–					3957
2-Aminoanthracene	DMSO	10	+	3950	6678	5408	2958	2712

DMSO, dimethyl sulphoxide; NC, not counted because of the precipitation.
Two plates were used for each dose and mean values of His^+ revertants per plate are indicated.

Table II. Mutagenicity of steviol in *S.typhimurium* TM677 with and without S9 activation

Compound	Dose (mg/ml)	S9	Survival[a] (per plate)	(%)	Mutants[b] (per plate)	Mutation frequency[c] ($\times 10^4$)
Steviol	0	–	85	100	7	0.8
	0.1	–	73	86	5	0.7
	0.5	–	73	86	7	1.0
	1.0	–	85	100	6	0.7
	2.5	–	84	99	9	1.1
	5.0	–	77	91	5	0.6
	7.5	–	84	99	8	1.0
	10.0	–	96	113	6	0.6
Steviol	0	+	170	100	55	3.2
	0.1	+	175	103	80	4.6
	0.5	+	100	59	102	10.2
	1.0	+	104	61	155	14.9
	2.5	+	77	45	269	34.9
	5.0	+	70	41	489	69.9
	7.5	+	70	41	641	91.6
	10.0	+	110	65	726	66.0
MNNG	0.0004	–	62	73	481	77.6
B[*a*]P	0.03	+	103	61	491	47.7

[a]After incubation for 1 h at 37°C, reaction mixtures were centrifuged and cell pellets resuspended in 1 ml phosphate-buffered saline. After dilution by 10^3, 0.1 ml of the diluted cell suspension was spread on agar plates, followed by incubation at 37°C for 2 days. Duplicate plates were used for determining the numbers of survivors and the mean values are indicated.
[b]A portion (0.1 ml) of the undiluted cell suspension was spread on agar plates containing 8-azaguanine and incubated for 2 days at 37°C. Duplicate plates were used and the mean values of mutants per plate are indicated.
[c]Mutation frequency was calculated as described in the Materials and methods section.
MNNG, *N-N'*-nitro-*N*-nitrosoguanidine; B[*a*]P, benzo[*a*]pyrene.

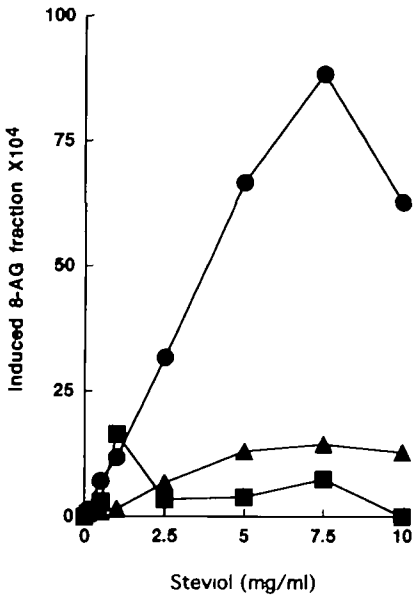

Fig. 1. Comparison of the mutagenicity of steviol with *S typhimurium* TM677, TM35 and KH75 in the presence of S9 mixture. ●, TM677 (*uvrB*, pKM101, *rfa*); ▲, TM35 (*uvrB*, *rfa*); ■, KH75 (*rfa*, pKM101).

Table III. Induction of umuC expression by stevioside and steviol in *S.typhimurium* TA1535/pSK1002

Compound	Dose (μg/ml)	S9	β-galactosidase activity (U/A_{600})[a]
Stevioside	0	–	36.9
	1250	–	39.2
	2500	–	41.9
	5000	–	45.0
	0	+	31.2
	1250	+	31 0
	2500	+	32.2
	5000	+	33 0
Steviol	0	–	37.1
	313	–	55 0
	625	–	74 1
	1250	–	73 2
	2500	–	53 6
	0	+	52.1
	625	+	57.2
	1250	+	96 0
	2500	+	99 9
Furylfuramide	0.03	–	1759
2-Aminoanthracene	3.3	+	1848

[a]Data represent mean values of duplicate determinations.

either with or without S9 mix (Table III). However, steviol induced an increase (~2-fold) in the specific activity of β-galactosidase at concentrations of 625–1250 μg/ml in the absence of S9 mix and 1250–2500 μg/ml in the presence of S9 mix. Under the conditions used, positive control chemicals, i.e. furylfuramide (0.03 μg/ml without activation) and 2-aminoanthracene (3.3 μg/ml with activation) substantially increased the specific activity of β-galactosidase of *S.typhimurium* TA1535/pSK1002. These results suggest that steviol is weakly positive in the *umu* test either with or without metabolic activation.

rec-assay

Neither stevioside nor steviol, either with or without S9 activation, inhibited the growth of *B.subtilis* H17 Rec^+ and M45 Rec^- (data not shown). The highest doses were 10 mg per paper disk. We also examined the genotoxicities of stevioside and steviol using the 'streak' *rec*-assay (Kada *et al.*, 1972). The two chemicals did not show any DNA damaging activities to *B.subtilis* either with or without S9 activation up to the concentration of 10 mg per paper disk (data not shown). Positive control chemicals, i.e. furylfuramide (0.01 μg without S9 activation) and 2-aminoanthracene (10 μg with S9 activation), inhibited the growth of *B.subtilis* M45 Rec^- to a greater extent than that of H17 Rec^+ in both spore and streak *rec*-assays. These results suggest that both stevioside and steviol are negative in the spore and streak *rec*-assays.

Chromosomal aberration test in vitro

In 24 and 48 h treatments without S9 mix, stevioside caused no significant increase in the frequencies of cells with chromosomal aberrations at doses up to 8.0 mg/ml. Cytotoxicity of the CHL cells was observed at 12.0 mg/ml both in 24 and 48 h treatments (Table IV). With S9 mix, it was negative at doses up to 12.0 mg/ml. Steviol was also negative at doses up to 0.5 mg/ml at 24 and 48 h treatments, without S9 mix (Table V). With S9 mix, on the other hand, steviol induced chromosomal aberrations at the dose range of 1.0–1.5 mg/ml (13–45% of aberrant cells). The majority of the aberrations were chromatid exchanges. The results indicate that steviol is clastogenic to cultured Chinese hamster cells only after metabolic activation.

Gene mutation assay of cultured mammalian cells

Treatment of CHL cells with steviol for 3 h in the presence of S9 mix induced a substantial dose-dependent increase in the number of mutants that were resistant to diphtheria toxin (Table VI). At the highest dose of 400 μg/ml, the frequency of DT^r mutants per survivors was about three times higher than that of the control. The mutagenicity of steviol on CHL cells in the presence of S9 mix is consistent with the results of preliminary experiments, in which steviol exhibited a dose-dependent increase in mutation frequency. The frequency was 110 DT^r-mutants per 2.5×10^5 survivors at a dose of 400 μg/ml. These results suggest that steviol is mutagenic to CHL cells in the presence of S9 mix. The mutagenicity of steviol without metabolic activation was not studied.

Micronucleus test

Steviol did not induce significant increases in the frequencies of MNPCEs, MNNCEs or MNEs at each treatment group (Table VII). No apparent changes were observed in the proportion of PCEs to total erythrocytes. At the highest dose of 1000 mg/kg, four out of six mice died, indicating that dose levels over the present maximum dose were unavailable for the micronucleus tests. Mitomycin C (2.0 mg/kg), a positive control, induced a significant increase in frequencies of MNPCEs and MNEs. These results suggest that steviol does not induce micronuclei in bone marrow erythrocytes of mice.

Discussion

To evaluate the genotoxic activities of stevioside and its aglycone, steviol, we carried out six *in vitro* and one *in vivo* mutagenicity assays. The results suggested that stevioside has no mutagenic or clastogenic potential in bacteria or cultured mammalian cells either with or without S9 activation (Table VIII). This is consistent with the previous reports that stevioside is negative with or without metabolic activation in mutagenicity tests using bacteria, cultured mammalian cells or insects

Table IV. Chromosomal aberration tests on stevioside using Chinese hamster cells in culture

Compound	Solvent	S9	Time (h)	Dose (mg/ml)	Polyploid (%)	Judge	Frequency of cells with chromosomal aberrations (%)							
							ctg	ctb	cte	frg	csb	cse	total	Judge
Stevioside	Saline	–	24–0	0	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	
				2.0	0.0	–	2.0	0.0	1.0	0.0	0.0	0.0	3.0	–
				4.0	0.0	–	1.0	1.0	0.0	0.0	0.0	0.0	2.0	–
				8.0	0.0	–	1.0	0.0	0.0	0.0	0.0	0.0	1.0	–
				12.0	NM		NM							
		–	48–0	0	1.0	–	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
				2.0	0.0	–	0.0	1.0	0.0	0.0	0.0	0.0	1.0	–
				4.0	0.0	–	0.0	1.0	0.0	0.0	0.0	0.0	1.0	–
				8.0	0.0	–	1.0	3.0	0.0	0.0	0.0	0.0	4.0	–
				12.0	TOX		TOX							
		+	6–18	0	2.0		3.0	0.0	0.0	0.0	0.0	0.0	3.0	
				2.0	0.0	–	1.0	1.0	1.0	0.0	0.0	0.0	3.0	–
				4.0	1.0	–	0.0	0.0	0.0	0.0	0.0	0.0	0.0	–
				8.0	0.0	–	0.0	1.0	0.0	0.0	0.0	0.0	1.0	–
				12.0	1.0	–	1.0	0.0	1.0	0.0	0.0	0.0	2.0	–
		–	6–18	0	1.0	–	0.0	2.0	0.0	0.0	0.0	0.0	2.0	
				2.0	0.0	–	0.0	1.0	0.0	0.0	0.0	0.0	1.0	–
				4.0	1.0	–	1.0	0.0	0.0	0.0	0.0	0.0	1.0	–
				8.0	1.0	–	1.0	0.0	1.0	0.0	0.0	0.0	2.0	–
				12.0	0.0	–	0.0	0.0	0.0	0.0	0.0	0.0	0.0	–

Time = treatment time – recovery time.
ctg, chromatid gaps (including chromosome gaps); ctb, chromatid breaks; cte, chromatid exchanges; frg, fragmentation; csb, chromosome breaks; cse, chromosome exchanges; NM, almost no metaphases; TOX, almost no surviving cells.

Table V. Chromosomal aberration tests on steviol using Chinese hamster cells in culture

Compound	Solvent	S9	Time (h)	Dose (mg/ml)	Polyploid (%)	Judge	Frequency of cells with chromosomal aberrations (%)							
							ctg	ctb	cte	frg	csb	cse	total	Judge
Steviol	CMC	–	24–0	0	2.0		1.0	0.0	0.0	0.0	0.0	0.0	1.0	
				0.125	0.0	–	1.0	1.0	0.0	0.0	0.0	0.0	2.0	–
				0.25	2.0	–	2.0	1.0	0.0	0.0	0.0	0.0	3.0	–
				0.5	1.0	–	3.0	1.0	0.0	0.0	0.0	0.0	4.0	–
		–	48–0	0	0		1.0	0.0	0.0	0.0	0.0	0.0	1.0	
				0.125	0.0	–	1.0	0.0	0.0	0.0	0.0	0.0	1.0	–
				0.25	1.0	–	1.0	0.0	0.0	0.0	0.0	0.0	1.0	–
				0.5	0.0	–	2.0	1.0	0.0	0.0	0.0	0.0	3.0	–
	DMSO	+	6–18	0	0.0		1.0	1.0	0.0	0.0	0.0	0.0	2.0	
				0.5	9.0	±	1.0	1.0	1.0	0.0	0.0	0.0	2.0	–
				0.75	0.0	–	2.0	0.0	7.0	0.0	0.0	0.0	9.0	±
				1.0	0.0	–	3.0	1.0	10.0	0.0	0.0	0.0	14.0	+
				1.25	0.0	–	4.0	6.0	30.0	0.0	0.0	0.0	40.0	+
				1.5	0.0	–	3.0	12.0	42.0	0.0	0.0	0.0	57.0	+
		–	6–18	0	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	
				0.5	0.0	–	0.0	0.0	0.0	0.0	0.0	0.0	0.0	–
				0.75	0.0	–	0.0	0.0	0.0	0.0	0.0	0.0	0.0	–
				1.0	0.0	–	1.0	1.0	1.0	0.0	0.0	0.0	3.0	–
				1.25	0.0	–	0.0	0.0	0.0	0.0	0.0	0.0	0.0	–
				1.5	0.0	–	1.0	0.0	0.0	0.0	0.0	0.0	1.0	–

Time = treatment time – recovery time.
ctg, chromatid gaps (including chromosome gaps); ctb, chromatid breaks; cte, chromatid exchanges; frg, fragmentation; csb, chromosome breaks; cse, chromosome exchanges; CMC, carboxymethylcellulose; DMSO, dimethyl sulphoxide.

(Okumura *et al.*, 1978; Medon *et al.*, 1982; Kawachi *et al.*, 1980; Kerr *et al.*, 1983; Pezzuto *et al.*, 1985; Nadamitsu *et al.*, 1985). It has also been reported that structurally related species, such as steviobioside, dulcoside A, rebaudioside A, rebaudioside B, rebaudioside C, isosteviol, dihydrosteviol A and dihydrosteviol B, are all negative in the forward mutation assay using *S.typhimurium* TM677 in the presence of S9 activation (Pezzuto *et al.*, 1985). In contrast, the aglycone, steviol, was positive in the forward mutation assay using *S.typhimurium* TM677, the *umu* test using *S.typhimurium* TA1535/pSK1002, the chromosomal aberration test and the gene mutation assay using CHL cells when S9 mix was present (Table VIII). The mutagenicity of steviol in the forward mutation assay is reported by Pezzuto *et al.* (1985). In the absence of S9 activation, the results of steviol were negative except for the *umu* test, in which it weakly increased the specific activity of β-galactosidase (Table III). Thus, we suggested that some metabolite(s) of steviol generated by S9 enzymes has genotoxic activities in bacteria and cultured mammalian cells.

Table VI. Cytotoxicity and mutagenicity of steviol on CHL cells in the presence of S9 activation

Compound	Dose (μg/ml)	Cytotoxicity[a] (colonies/dish)	(%)	DT^r colonies[b] per dish	Plating efficiency[c] (colonies/dish)	(%)	DT^r colonies per 2×10^5 survivors[d]
Steviol	0	324	100	26	170	68	38
	250	282	87	33	178	71	46
	300	213	66	19	148	59	32
	350	98	30	35	157	63	56
	400	11	3	86	168	67	128
DMN[e]	1000	6	2	110	132	53	208

[a]Cytotoxicity was determined by culturing steviol-treated cells (200–400 cells) for 7 days and counting the numbers of surviving colonies.
[b]The number of diphtheria toxin-resistant colonies were determined by culturing the treated cells (2.5×10^5 cells) in medium containing diphtheria toxin for 7 days and counting the number of surviving colonies
[c]Plating efficiency was determined by culturing the treated cells (250 cells) in toxin-free medium for 7 days and counting the surviving colonies.
[d]The number of diphtheria toxin-resistant colonies per 2×10^5 survivors was calculated by dividing the number of diphtheria toxin resistant colonies per dish by 1/100 of the percent of plating efficiency
[e]*N*, *N*-dimethylnitrosamine; DT, Diptheria toxin.

Table VII. Micronucleus test with mice treated with steviol

	Dose (mg/kg)	Treatment	MNPCE (%)	MNNCE (%)	MNE (%)	PCE (%)
Olive oil		1×24	0.60 + 0.24[a]	0.48 + 0.24	0.52 + 0.12	49.68 + 3.8
	125	1×24	0.45 + 0.08	0.35 + 0.31	0 40 + 0 21	54.23 + 3.21
	250	1×24	0.52 + 0.27	0.35 + 0.08	0.37 + 0.23	48.83 + 5.2
	500	1×24	0 38 + 0 16	0.25 + 0 15	0.28 + 0.12	46.72 + 3.05
		1×48	0.35 + 0.13	0 23 + 0.10	0.25 + 0.13	54.33 + 7 82
	1000	1×24	0.55 + 0 49	0 35 + 0.07	0.50 + 0.42	50 15 + 4 74
MMC	2 0	1×24	14.88 + 2.55	0.35 + 0.28	6.52 + 1 35	46.23 + 2.78

[a]Mean + SD.
MNPCE, frequency of micronucleated polychromatic erythrocytes; MNNCE, frequency of micronucleated normochromatic erythrocytes; MNE, frequency of micronucleated erythrocytes; PCE, ratio of polychromatic erythrocytes to total erythrocytes

Table VIII. Summary of the results of genotoxicity tests of stevioside and steviol

Test	Stevioside		Steviol	
	– S9 mix	+ S9 mix	– S9 mix	+ S9 mix
Reverse mutation test	–	–	–	–
Forward mutation test	–	–	–	+
umu test	–	–	+w	+w
Rec assay	–	–	–	–
Chromosome aberration test	–	–	–	+
Gene mutation test	NT	NT	NT	+
Micronucleus test				–

+w, weakly positive; NT, not tested.

The primary metabolic pathways of steviol catalyzed by S9 enzymes involve allylic oxidation and epoxidation (Compadre *et al.*, 1988). The possibility that the active metabolite is an epoxide is ruled out because epoxide hydrolase does not inhibit steviol-induced mutagenicity (Pezzuto *et al.*, 1986). Compadre *et al.* (1988) proposed that 15-oxosteviol, an oxidative metabolite of 15-hydroxysteviol, is the active metabolite, since it induces mutations that confer 8-azaguanine resistance in *S.typhimurium* TM677 without further metabolic activation. However, Procinska *et al.* (1991) could not confirm the direct-acting mutagenicity of 15-oxosteviol and suggested that the conclusion by Compadre *et al.* was due to misinterpretation of the data. Thus, at this moment, the active metabolite(s) of steviol that causes mutations in *S.typhimurium* TM677 remains to be identified.

Procinska *et al.* (1991) also suggested that the observed mutagenicity with steviol might be due to an impurity, because the mutagenicities of the steviol solution increase as the amount added to the solution increases up to 10 mg/ml, whereas the concentration of steviol is limited to its saturation level of ~1 mg/ml. Although we can not rule out the possibility, we do not think it is likely, because we have confirmed the positive results of Pezzto *et al.* (1986) with different preparations of steviol. Also, secondly some substances, such as polycyclic hydrocarbons, that are very insoluble in aqueous media still exhibit increasing genotoxic responses with dose above the solubility limit (Kirkland, 1994).

Although steviol was mutagenic and clastogenic in bacteria and cultured mammalian cells, it did not exhibit any positive response in the mouse micronucleus test (Table VII). This *in vivo* test result does not necessarily mean that neither mutagenic nor clastogenic metabolites are generated from steviol *in vivo*. It could be possible that steviol produced adverse metabolites *in vivo* but they did not reach the bone marrow, the target organ for the micronucleus test. In fact, dimethylnitrosamine and diethylnitrosamine, potent hepatocarcinogens, do not give rise to a substantial increase in the number of micronucleated cells in mouse micronucleus test, probably because the short-lived active metabolites generated in the liver can not reach the bone marrow (Proudlock and Allen, 1986; Cliet *et al.*, 1993). It might also be possible that the genotoxic metabolites of steviol could reach bone marrow but that they predominantly induced point mutations, such as base change or frameshifts, rather than chromosome aberrations, so that no micronucleated blood cells were found in the steviol-treated mice. Thus, further work is necessary to predict the genotoxic risk of steviol to human beings. Since steviol

requires S9 activation for mutagenesis and clastogenesis *in vitro*, the genotoxic damage in the liver of rats or mice should be examined. In this respect, the liver unscheduled DNA synthesis (UDS) assay or a transgenic mutagenicity assay would be appropriate for the further assessment of the genotoxic potential of steviol *in vivo*.

In summary, we examined the mutagenicity and clastogenicity of stevioside and its aglycone, steviol, with *in vitro* and *in vivo* mutagenicity tests. Although stevioside was negative in all of the assays examined, steviol gave rise to an increase in *gpt* gene mutations in *S.typhimurium* TM677, in chromosome aberrations and DT^r gene mutations in CHL cells when S9 mix was present. Although the number of micronucleated cells in bone marrow erythrocytes did not increase in steviol-treated mice, the present *in vitro* test results suggested the necessity of other *in vivo* mutagenicity tests such as liver UDS assay or transgenic mouse mutagenicity test for more precise assessment of genotoxic risk of steviol to human beings.

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