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Genotoxicity testing of a *Salacia oblonga* extract

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Abstract

Salacia oblonga has been used for thousands of years in Ayurvedic medicine for the oral treatment of diabetes. The root extract has been shown to inhibit the activity of intestinal α-glucosidases, therefore *S. oblonga* holds potential as a natural method to mitigate the blood glucose response for people with diabetes. As part of a safety evaluation of novel ingredients for use in blood glucose control, the potential genotoxicity of a *S. oblonga* root extract (SOE) was evaluated using the standard battery of tests (reverse mutation assay; chromosomal aberrations assay; mouse micronucleus assay) recommended by US Food and Drug Administration (FDA) for food ingredients. SOE was determined not to be genotoxic under the conditions of the reverse mutation assay and mouse micronucleus assay, and weakly positive for the chromosomal aberrations assay. A reproducible, although weak, positive chromosomal aberrations response in human lymphocytes is of concern and further toxicity research is recommended. Use of SOE is presently expected to be safe, as anticipated intake is small compared to the doses administered in the genotoxicity assays and may, after further toxicity research, may prove be a useful ingredient in foodstuffs.

Keywords: *Salacia oblonga*; α-Glucosidase inhibitor; Toxicology; Safety; Reverse mutation assay; Chromosomal aberration assay; Mouse micronucleus assay

1. Introduction

Salacia oblonga is a perennial wild, woody, climbing vine native to India and Sri Lanka. A member of the *Celastaceae* family, it is commonly known as "ponkoranti" due to its golden colored root bark. *S. oblonga* has been used for thousands of years in Ayurvedic medicine for the oral treatment of diabetes (Grover et al., 2002). Root extracts of various *Salacia* species have been shown to inhibit the activity of intestinal α-glucosidases (Matsuda et al., 2005). Inhibition of the carbohydrate metabolizing enzymes results in delayed breakdown of oligosaccharides and inhibits glucose absorption into the bloodstream. Decreased glucose absorption results in a lower postprandial glycemic response and improved overall glycemic control. Extract constituents salacinol, kotalanol, and mangiferin have been characterized as the active ingredients for blood glucose control by inhibition of enzymes involved in glycemic response (Ghavami et al., 2001; Matsuda et al., 1999; Matsuda et al., 2002; Miura et al., 2001). Therefore, *S. oblonga* holds potential as a natural method to mitigate the blood glucose response for people with diabetes. As an ingredient incorporated into foodstuff, *S. oblonga* may provide people with diabetes a convenient way to help manage their blood glucose levels.

As part of a safety evaluation of novel ingredients for use in blood glucose control, an evaluation of the potential genotoxicity of a *S. oblonga* extract (SOE) was conducted using the standard battery of tests recommended by FDA for food ingredients. The tests included the bacterial reverse mutation assay, the chromosomal aberrations assay, and the mouse micronucleus assay.

Abbreviations: FDA, US Food and Drug Administration; ICH, International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use; SOE, *Salacia oblonga* extract.

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2. Materials and methods

2.1. Chemicals, culture medium and S9 activation system

Cell culture grade water used in the mouse micronucleus assay was purchased from BioWhittaker. Benzo[*a*]pyrene, cyclophosphamide (CP), mitomycin C, sodium azide, and ICR-191 were purchased from Sigma Chemical Co. 2-aminoanthracene and 2-nitrofluorene were obtained from Aldrich Chemical Co. 4 nitroquinoline N oxide (4NQO) was obtained from Supleco. Dimethylsulfoxide was obtained from Acros Organics. Cell culture grade water used in the chromosomal aberration assay and the reverse mutation assay, culture medium, antibiotics and L-glutamine were obtained from Mediatech.

S9 liver homogenate was purchased from Molecular Toxicology, Inc. The homogenate was prepared from male Sprague–Dawley rats pretreated with Aroclor™ 1254 as described by Ames et al. (1975). For the reverse mutation assay, the S9 mix consisted of 10% S9, 4 mM NADP, 5 mM glucose-6-phosphate, 8 mM $MgCl_2$, 33 mM KCl, and 100 mM sodium phosphate, pH 7.4.

Top (overlay) agar for the reverse mutation assay was prepared with 0.7% (w/v) agar and 0.5% (w/v) NaCl and was supplemented with 10 mL of (1) 0.5 mM histidine/biotin solution per 100 mL agar for selection of histidine revertants, or (2) 0.5 mM tryptophan solution per 100 mL of agar for selection of tryptophan revertants.

S. oblonga extract D (Lot# 050203) was obtained in powder form from Tanabe USA, Inc.

2.2. Cells

Salmonella typhimurium histidine auxotrophs TA98, TA100, TA1535 and TA1537 were obtained from Dr. Bruce Ames, University of California, Berkeley. The *Escherichia coli* tryptophan auxotroph WP2*uvr*A was received from the National Collection of Industrial Bacteria, Torrey Research Station, Scotland (United Kingdom). The tester strains were checked for retention of their characteristic phenotypic markers at the time of use.

2.3. Salmonella–E. coli/mammalian-microsome assay

The bacterial reverse mutation assay, to evaluate the ability to induce reverse mutations at the histidine loci in four strains of *S. typhimurium* (TA98, TA100, TA1535 and TA1537) and at the tryptophan locus in *E. coli* tester strain WP2*uvr*A, was conducted according to standard procedures (Ames et al., 1975; Green and Muriel, 1976). Briefly, the tester strains were exposed to SOE via the plate incorporation method in the presence and absence of an exogenous metabolic activation system (S9). SOE, tester strain, and S9 mix (when required) were added to molten top agar supplemented with histidine and biotin or tryptophan. The mixture was vortexed and overlaid onto the surface of 25 mL of minimal bottom agar contained in a 15×100 mm petri dish. After the overlay solidified, the plates were inverted and incubated for 52 ± 4 h at 37 ± 2 °C. All doses of the test article, the vehicle controls and the positive controls were plated in triplicate. After incubation, the revertant colonies were counted. A mutagenic response was characterized by at least a 2-fold (tester strains TA98, TA100 and WP2*uvr*A) or 3-fold (TA1535 and TA1537) dose-dependent increase in the mean revertants per plate of at least one of these tester strains as compared to the concurrent vehicle control.

2.4. Chromosomal aberrations assay

Human venous blood from healthy, adult donors (nonsmokers without a history of radiotherapy, chemotherapy or drug usage, and lacking current viral infections) was used in the chromosomal aberrations assay, conducted to evaluate the ability to cause structural chromosomal aberrations with and without exogenous metabolic activation system. Whole blood cultures containing fresh heparinized blood, culture medium and test article were incubated at 37 °C ± 2 °C in a humidified atmosphere of 5% ± 1.5% CO_2 in air. The medium was RPMI 1640 supplemented with HEPES buffer (25 mM), ~20% heat-inactivated fetal bovine serum (FBS), penicillin (100 mL), streptomycin (100 μg/mL), L-glutamine (2 mM) and 2% phytohemagglutinin M (PHA-M). Negative (untreated controls) and vehicle controls (cultures treated with 10.0 μL of DMSO/mL) were used. The positive control agents were mitomycin C (MMC) for the nonactivation series and CP in the metabolic activation series.

The *in vitro* metabolic activation system (Maron and Ames, 1983) consisted of a rat liver post-mitochondrial fraction (S9) and an energy-producing system (NADP at 1.5 mg/mL (1.8 mM) and isocitric acid at 2.7 mg/mL (10.5 mM)). S9 was prepared five days after a single dose of 500 mg/kg of Aroclor™ 1254.

In the initial trial, cultures were treated for ~3 h with and without S9 and harvested ~22 h after initiation of treatment. In the second trial, cultures were treated for ~22 h without S9 and ~3 h with S9 and harvested ~22 h after initiation of treatment, corresponding to 1.5 times a cell cycle time of approximately 15 h after the lymphocytes are induced to divide by the addition of PHA-M (Galloway et al., 1994). At harvest, cells were swollen with 75 mM KCl hypotonic solution and fixed with absolute methanol:glacial acetic acid (3:1, v/v) fixative. One hundred cells from each duplicate culture were analyzed for the different types of chromosomal aberrations (Evans, 1962, 1976). Mitotic index, percent polyploidy and endoreduplication were evaluated from the negative control, vehicle control and a range of test article concentrations and used for measurement of toxicity and selection of doses for analysis. Statistical analysis employed a Cochran–Armitage test for linear trend and Fisher's Exact Test (Thakur et al., 1985).

2.5. In vivo mouse micronucleus assay

Healthy male Crl:CD -1(ICR)BR mice (8–10 weeks of age; Harlan Sprague-Dawley, Inc.) were used in the micronucleus assay to evaluate the ability to induce *in vivo* clastogenic activity and/or disruption of the mitotic apparatus by detecting micronuclei in polychromatic erythrocyte (PCE) cells in mouse bone marrow. Mice were dosed by oral gavage for three consecutive days at 500, 1000 or 2000 mg/kg/day SOE, or the vehicle control (cell culture grade water; five/group, approximately 24 h between doses). A similar group was treated once with the positive control (80 mg/kg cyclophosphamide) approximately 24 h prior to sacrifice.

Bone marrow was harvested from five mice per group approximately 24 h after the last dose. At least 2000 PCEs per animal were analyzed for the frequency of micronuclei. Cytotoxicity was assessed by scoring the number of PCEs and normochromatic erythrocytes (NCEs) in at least the first 500 erythrocytes for each animal. The criteria for the identification of micronuclei were those of Schmid (1976). Data analysis was performed using an analysis of variance (Winer, 1971) on untransformed proportions micronucleated cells per animal, and on untransformed PCE:NCE ratios when the variances were homogeneous.

3. Results

3.1. Salmonella–E. coli/mammalian-microsome assay

The mutagenicity of SOE in bacteria was evaluated up to a maximal dose of 5000 μg/plate. Normal growth was observed in all five tester strains, and the test article was freely soluble at all doses evaluated with and without S9. No increases in revertant frequencies were observed at any dose of SOE in any tester strains with or without S9 compared to the concurrent vehicle control cultures (Table 1). The test article was re-evaluated in an independent confirmatory experiment under identical conditions, and similar results were observed (Table 2).

Table 1
Bacterial reverse mutation assay initial results

	Dose μg/Plate	Mean revertants per plate with standard deviation									
		TA98		TA100		TA1535		TA1537		WP2*uvr*A	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
+*S*9											
Vehicle Control[a]		28	7	97	15	11	4	6	1	14	3
SOE	33.3	22	6	112	17	17	1	4	3	14	5
	100	21	3	86	10	11	5	8	2	8	3
	333	31	3	104	5	9	1	9	4	11	3
	1000	14	7	99	10	11	6	8	1	14	4
	3330	17	10	84	10	8	1	6	3	7	1
	5000	17	2	87	7	7	4	4	2	10	5
Benzo[*a*]pyrene	2.5	275	48								
2-Aminoanthracene	2.5			756	89	110	16	142	27		
2-Aminoanthracene	25.0									292	101
−*S*9											
Vehicle Control[a]		8	2	70	3	9	1	5	3	14	3
SOE	33.3	7	2	77	6	11	3	5	3	15	1
	100	10	2	80	5	12	4	5	2	12	3
	333	12	4	69	6	12	4	5	3	14	3
	1000	10	0	63	4	9	1	4	2	13	7
	3330	9	2	64	10	15	6	6	2	9	1
	5000	11	3	64	7	16	7	4	5	9	1
2-Nitrofluorene	1.0	339	6								
Sodium azide	2.0			1404	395	856	30				
ICR-191	2.0							904	87		
4-Nitroquinoline-*N*-oxide	1.0									346	70

[a] Vehicle control = DMSO, 100 μL aliquot.

Table 2
Bacterial reverse mutation assay confirmatory results

	Dose μg /Plate	Mean revertants per plate with standard deviation									
		TA98		TA100		TA1535		TA1537		WP2*uvr*A	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
+*S*9											
Vehicle Control[a]		23	3	98	8	13	4	12	5	16	2
SOE	33.3	21	7	95	11	12	4	15	4	15	4
	100	23	3	107	6	13	3	13	4	17	1
	333	22	4	95	4	9	1	13	3	11	3
	1000	24	5	96	8	10	2	10	5	14	3
	3330	20	5	89	12	11	4	9	2	13	2
	5000	17	6	90	19	10	1	9	1	9	2
Benzo[*a*]pyrene	2.5	302	25								
2-Aminoanthracene	2.5			571	67	158	81	97	11		
2-Aminoanthracene	25.0									704	80
−*S*9											
Vehicle Control		12	3	90	2	11	2	7	2	15	4
SOE	33.3	14	6	88	15	10	2	8	2	16	3
	100	12	7	87	8	7	4	8	2	18	7
	333	17	5	82	16	10	5	6	4	21	6
	1000	14	5	85	10	13	5	11	3	12	6
	3330	10	3	89	13	7	2	11	2	9	3
	5000	11	5	81	11	9	5	9	2	10	6
2-Nitrofluorene	1.0	292	60								
Sodium azide	2.0			946	76	780	111				
ICR-191	2.0							803	22		
4-Nitroquinoline-*N*-oxide	1.0									339	40

[a] Vehicle control = DMSO, 100 μL aliquot.

Table 3
Chromosomal aberration assay results without metabolic activation

		Dose (μg/mL)	# Cells scored for aberrations	# Cells scored for pp and er	% of pp Cells	% of er Cells	Judgement (+/−)[a]	Percentages of cells showing structural chromosome aberrations							Judgement (+/−)[c]
								Gaps	Simple breaks	chte	chre	Mab	Totals[b]		
													−g	+g	
Initial trial without metabolic activation															
Controls															
Negative:	RPMI 1640		200	200	0.5	0.0		0.5	0.5				0.5	1.0	
Vehicle:	DMSO	10.0[d]	200	200	0.0	0.0		3.5	2.0				2.0	5.5	
Positive:	MMC	1.00	100	200	0.0	0.0	−	7.0	45.0	23.0			58.0	60.0	+
SOE		353	200	200	0.5	0.0	−	2.5	3.5				3.5	6.0	−
		504	200	200	0.5	0.0	−	2.5	3.0	0.5			3.5	5.5	−
		1030	200	200	6.5	0.0	+	5.0	5.0				5.0	9.0	−
		1470	200	200	3.5	0.0	−	6.0	6.0				6.0	10.0	−
Confirmatory trial without metabolic activation															
Controls															
Negative:	RPMI 1640		200	200	0.0	0.0		1.0					0.0	1.0	
Vehicle:	DMSO	10.0[d]	200	200	0.0	0.0		0.5	0.5				0.5	1.0	
Positive:	MMC	0.300	100	200	0.0	0.0	−	4.0	35.0	8.0			41.0	44.0	+
SOE		150	200	200	1.5	0.0	−	2.0	0.5				0.5	2.5	−
		225	300	200	0.5	0.0	−	7.7	4.3		0.3		4.7	11.0	+
		300	300	200	0.0	0.0	−	10.3	9.7	0.3			10.0	18.7	+
		450	300	200	0.0	0.0	−	8.7	4.0				4.0	11.3	−

chte: chromatid exchange chre: chromosome exchange mab: multiple aberrations, greater than 4 aberrations pp: polyploidy er: endoreduplication.

[a] Significantly greater in % polyploidy than the vehicle control, $p \leqslant 0.01$.

[b] $-g$ = # or % of cells with chromosome aberrations; $+g$ = # or % of cells with chromosome aberrations + # or % of cells with gaps.

[c] Significantly greater in $-g$ than the vehicle control, $p \leqslant 0.01$.

[d] μL/mL RPMI 1640 = culture medium; DMSO = dimethylsulfoxide; MMC = Mitomycin C.

Table 4
Chromosomal aberration assay results with metabolic activation

		Dose (μg/mL)	# Cells scored for aberrations	# Cells scored for pp and er	% of pp Cells	% of er cells	Judgement (+/−)[a]	Percentages of cells showing structural chromosome aberrations							Judgement (+/−)[c]
								Gaps	Simple breaks	chte	chre	Mab	Totals[b]		
													−g	+g	
Initial trial with metabolic activation															
Controls															
Negative:	RPMI 1640		200	200	0.0	0.0		1.0	1.0				1.0	2.0	
Vehicle:	DMSO	10.0[d]	200	200	0.0	0.0		1.0	1.5				1.5	2.5	
Positive:	CP	25.0	100	200	0.0	0.0	−	7.0	37.0	9.0		3.0	47.0	52.0	+
SOE		504	200	200	0.0	0.0	−	2.0	2.5				2.5	4.5	−
		720	200	200	0.5	0.0	−	2.0	1.5				1.5	3.5	−
		1030	200	200	1.5	0.0	−	2.5	3.0	0.5			3.0	5.0	−
		1470	200	200	7.0	0.0	+	4.5	4.5				4.5	8.5	−
Confirmatory trial with metabolic activation															
Controls															
Negative:	RPMI 1640		200	200	0.0	0.0		2.0					0.0	2.0	
Vehicle:	DMSO	10.0[d]	200	200	0.0	0.0		0.5					0.0	0.5	
Positive:	CP	25.0	100	200	0.0	0.0	−	3.0	42.0	8.0			44.0	46.0	+
SOE		300	200	200	0.0	0.0	−	3.0	0.5				0.5	3.5	−
		600	300	200	0.0	0.0	−	1.0	2.5				2.5	3.5	−
		1000	300	200	1.5	0.0	−	7.8	5.3		0.5		5.8	12.3	+
		1500	300	200	5.5	0.0	+	4.5	3.0				3.0	6.5	−

chte: chromatid exchange chre: chromosome exchange mab: multiple aberrations, greater than 4 aberrations pp: polyploidy er: endoreduplication.

[a] Significantly greater in % polyploidy than the vehicle control, $p \leqslant 0.01$.

[b] $-g$ = # or % of cells with chromosome aberrations; $+g$ = # or % of cells with chromosome aberrations + # or % of cells with gaps.

[c] Significantly greater in $-g$ than the vehicle control, $p \leqslant 0.01$.

[d] μL/mL RPMI 1640 = culture medium; DMSO = dimethylsulfoxide; CP = Cyclophosphamide.

Table 5
In vivo Micronucleus assay results

Treatment	Dose[a]	% Micronucleated PCEs mean of 2000 per animal ± S.E. Males	Ratio PCE:NCE[b] Mean ± S.E. Males
Controls			
Water	10 mL/kg/day	0.08 ± 0.02	0.62 ± 0.08
	80 mg/kg	3.03 ± 0.42[c]	0.38 ± 0.07
Cyclophosphamide			
SOE	500 mg/kg/day	0.09 ± 0.02	0.59 ± 0.10
	1000 mg/kg/day	0.07 ± 0.02	0.59 ± 0.08
	2000 mg/kg/day	0.05 ± 0.02	0.69 ± 0.10

[a] Animals were treated for three consecutive days (water and SOE) or once (cyclophosphamide) and sacrificed 24 h after the last dose. Five males per group were analyzed.
[b] At least 2000 PCEs per animal were analyzed for the frequency of micronuclei. Cytotoxicity was assessed by scoring the number of PCEs and normochromatic erythrocytes (NCEs) in at least the first 500 erythrocytes for each animal.
[c] Significantly greater than the corresponding vehicle control, $p \leqslant 0.01$.

3.2. Chromosomal aberrations assay

The highest concentration tested in the chromosomal aberrations assay, 3000 μg/mL, was above the solubility limit of the test article after dosing into culture medium. In the initial chromosomal aberrations assay, the treatment period was for ~3 h with and without metabolic activation, and in the confirmatory chromosomal aberrations assay, the treatment period was ~22 h without metabolic activation and ~3 h with metabolic activation. Cultures were harvested ~22 h from the initiation of treatment. SOE induced slight increases in structural and numerical chromosomal aberrations (Tables 3 and 4).

3.3. In vivo mouse micronucleus assay

SOE did not induce signs of clinical toxicity, or evidence of bone marrow cytotoxicity (i.e., no statistically significant decrease in the PCE:NCE ratios), at doses up to 2000 mg/kg/day. In addition, SOE did not induce any statistically significant increases in micronucleated PCEs at any dose examined (Table 5).

4. Discussion

Genotoxicity testing conducted on SOE followed the standard 3-test battery recommended by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and by FDA. Negative results in the bacterial reverse mutation and mouse micronucleus assays demonstrate that SOE is devoid of any significant genotoxic activity under the conditions of the assays. Weakly positive results were observed in the human peripheral blood chromosomal aberration assay at SOE concentrations with and without precipitate. However, there was no clear or consistent dose-related response observed. Therefore, the possible biological relevance of the positive response cannot be assessed without additional work.

The existence of a reproducible, although weak, positive chromosomal aberrations response in human peripheral blood lymphocytes is of concern. Potential next steps include, but are not limited to: a Syrian Hamster Embryo Cell Transformation Assay, a mouse p53 transgenic assay, an analysis of peripheral blood smears for micronuclei in normochromatic erythrocytes from a repeat dose mouse toxicity study, a DNA adduct analysis, or a rat or monkey peripheral blood lymphocyte chromosomal aberrations assay added on at the termination of a subchronic or chronic repeat dose toxicity study. For SOE, the latter is recommended. The possible assays to further investigate the *in vitro* positive result observed with SOE are detailed in the FDA Guidance for Industry and Review Staff Document (January, 2006).

Use of SOE is presently expected to be safe, as anticipated intake is small compared to the doses administered in the genotoxicity assays. The *S. oblonga* extract SOE, after further toxicity research, may prove be a useful ingredient in foodstuffs to help mitigate the blood glucose response for people with diabetes.

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