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Original Article

Genotoxicity of Monascus Color Y-001

Ryosuke Sato[1], Michihito Takabe[1], Takahiro Ishii[2], Norio Imai[3], Yuko Doi[3] and Toyohiko Aoki[3]

[1]Tokyo Laboratory, BoZo Research Center Inc., 1-3-11, Hanegi, Setagaya-ku, Tokyo 156-0042, Japan
[2]Gotemba Laboratory, BoZo Research Center Inc., 1284, Kamado, Gotemba-shi, Shizuoka 412-0039, Japan
[3]DIMS Institute of Medical Science, Inc., 64 Goura, Nishiazai, Azai-cho, Ichinomiya, Aichi, 491-0113, Japan

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ABSTRACT — The genotoxic potential of Monascus Color Y-001 was assessed using the standard battery of assays including the *in vitro* reverse mutation test in bacteria (Ames test), the *in vitro* chromosomal aberration test in mammalian cells and the *in vivo* micronucleus test in rats. The results of the Ames test, the chromosomal aberration test in CHL/IU cells with and without S9 mix (metabolic activation) and the *in vivo* bone marrow micronucleus test in rats were all negative. Therefore, it is concluded that Monascus Color Y-001 does not possess any genotoxic risk in humans.

Key words: Monascus Color Y-001, Genotoxicity, Ames test, Chromosomal aberration test, Micronucleus test

INTRODUCTION

Monascus Color Y-001, one of the Monascus colors produced from *Monascus purpureus* Y-001 fermentation, is a natural food dye that is widely used in food industries, especially in Japan (Feng *et al.*, 2012). The toxicological effects of Monascus Color Y-001 have been investigating, and in this paper genotoxicity study results are reported as one of nonclinical safety assessment of Monascus Color Y-001. Monascus Color Y-001 was evaluated for genotoxic potential in both *in vitro* and *in vivo* assays. Potential mutagenic activity was assessed in bacteria *in vitro* by the standard bacterial reverse mutation test (Ames test). The genotoxic potential of Monascus Color Y-001 was assessed by the *in vitro* chromosomal aberration test using Chinese hamster lung fibroblast cells (CHL/IU). These *in vitro* assays were carried out in both the presence and absence of an exogenous metabolizing system (S9 mix). The potential for clastogenic effects was also assessed by an *in vivo* assay using the oral micronucleus test in rats.

MATERIALS AND METHODS

All studies were conducted at Gotemba Laboratory, BoZo Research Center Inc. in compliance with Good Laboratory Practice (GLP) regulations and in accordance with all relevant guidelines, including the Redbook 2000 guidelines (FDA) and the OECD Guidelines for the Testing of Chemicals. The *in vivo* micronucleus assay was conducted at a test facility with Full Accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International and under the approval of the Institutional Animal Care and Use Committee (IACUC) of the test facility.

Test article

The test article, Monascus Color Y-001, was supplied by DIMS Institute of Medical Science, Inc. Monascus Color Y-001 (Manufacturer: YAEGAKI Bio-industry, Inc., Hyogo, Japan) used in the *in vitro* and *in vivo* studies was a solid substance containing 55.8% of N-Leucyl monas-

Correspondence: Toyohiko Aoki (E-mail: aoki@dims.co.jp)

corubrin (M-Leu) and 42.2% of N-Leucyl rubropunctatin (R-Leu), and the stability during the experimental period was verified by post-analysis. Dose formulations were prepared by dissolving Monascus Color Y-001 in water for injection for the two *in vitro* assays and 0.1 w/v% polyoxyethylene sorbitan monooleate (0.1% Tween 80) for the *in vivo* micronucleus assay. In each study, dose analyses demonstrated that the dose formulations had achieved target concentrations.

Bacterial reverse mutation test (Ames test)

Salmonella typhimurium TA100 and TA1535 and *Escherichia coli* WP2 *uvrA* were used as the base-pair substitution types, and *Salmonella typhimurium* TA98 and TA1537 as the frame-shift type. Bacteria were obtained from the Division of Genetics and Mutagenesis, National Institute of Health Sciences (Tokyo, Japan). The assay was performed by the pre-incubation method. The following 5 positive control articles were used: 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2), 2-methoxy-6-chloro-9-[3-(2-chloroethyl)-aminopropylamino]acridine·2HCl (ICR-191), 2-aminoanthracene (2AA), benzo[*a*]pyrene (B[*a*]P) and sodium azide (SAZ). AF-2, 2AA, SAZ and B[*a*]P were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) and ICR-191 from SIGMA-Aldrich Co. LLC (Oakville, Ontario, Canada). As a metabolic activation system, S9 mix was prepared by mixing rat liver S9 fraction, which was induced by treatment with phenobarbital and 5,6-benzoflavone, and cofactor.

Prior to the main test, the range-finding test was conducted to investigate preliminarily growth inhibition and reverse mutation potential at 5000, 1250, 313, 78.1 and 19.5 μg/plate in all tester strains with or without metabolic activation. The dose levels in the main test were set on the basis of the range-finding test. In addition to the test article group, a concurrent negative control group and a concurrent positive control group were provided. The test procedures are described briefly in the following. An aliquot of the dose formulation, vehicle or positive control solution was separately put into a sterilized test tube, then S9 mix or 0.1 mol/L phosphate buffer was added, and the bacterial suspension was added. After incubation for 20 min at 37°C while shaking, molten top agar was added to each test tube, mixed and overlaid uniformly onto the surface of a minimal glucose agar plate. All plates were incubated for approximately 48 hr at 37°C. After incubation, all plates were observed for growth condition under a stereomicroscope and examined visually for the presence/absence of precipitates. The number of revertant colonies was counted using a dot counter.

When the results fulfill either of the following criteria, the test article is judged positive: i) the mean number of revertant colonies in the test article group in at least 1 tester strain is 2-fold or more that in the concurrent negative controls with dose-dependency or reproducibility, ii) the mean number of revertant colonies in the test article group increase markedly compared with that in the concurrent negative control, even if there is no clear dose-response.

In vitro chromosomal aberration test

Chinese hamster lung fibroblast cells (CHL/IU) obtained from National Institute of Biomedical Innovation (Osaka, Japan) were used. The culture medium (10% BS-MEM) was prepared by adding inactivated bovine serum (BS, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 10 v/v% to Minimum Essential Medium (MEM, Thermo Fisher Scientific Inc.). The following 2 positive control articles were used in the present study: Mitomycin C (MMC, Kyowa Kirin Co., Ltd., Tokyo, Japan) and Cyclophosphamide monohydrate (CP, FUJIFILM Wako Pure Chemical Corporation). As a metabolic activation system, S9 mix was prepared by mixing rat liver S9 fraction, which was induced by treatment with phenobarbital and 5,6-benzoflavone, and cofactor. The experiment was composed of three treatment schedules: 1) short-term treatment without metabolic activation (6 hr-treatment with test/control article without S9 mix followed by 18 hr-recovery culture in the 10% BS-MEM medium), 2) short-term treatment with metabolic activation (6 hr-treatment with test/control article with S9 mix followed by 18 hr-recovery culture in the 10% BS-MEM medium), 3) continuous treatment (24 hr-treatment with test/control article without S9 mix). Prior to the main test, a range-finding test was conducted to investigate preliminarily growth inhibition potential at 5000, 2500, 1250, 625, 313, 156, 78.1, and 39.1 μg/mL. The dose levels in the main test for each treatment schedule were set on the basis of the range-finding test. In the main test, a concurrent negative control group and a concurrent positive control group were provided for each treatment schedule. Plastic plates (Petri dishes of 60 mm diameter) were used for incubation. Triplicate cultures were performed in the main test (two for preparing chromosome slides, and one for determining cell density at the end of incubation). The cells (2×10^4) were seeded and cultured for 3 days. After that, the cells were incubated with the test or control article, S9 mix or vehicle according to the respective treatment schedule. After incubation, the cells were harvested after

treatment with 0.25% trypsin from three cultures, one for counting cell density to calculate Population Doubling (PD) and Relative Population Doubling (RPD), and two for chromosome assay. The cells from two cultures were treated with hypotonic 0.075 M potassium chloride for approximately 15 min, and fixed with Carnoy's fixative. The fixed cell suspension was dropped onto 2 places on a glass slide, air-dried and stained with Giemsa solution to prepare chromosome slides.

Well-spread metaphase chromosomes, 300 per test group (150 cells per culture), were scored under a microscope and the types of structural aberrations and the number of cells with abnormalities were recorded. The frequency of polyploidy was also recorded. All slides were analyzed by the blind method. Four dose levels in each treatment schedule including a dose producing cytotoxicity without interfering with observation by precipitates whose RPD is 45% ± 5% and doses showing moderate and little or no cytotoxicity were observed. Chromosomal aberrations were classified broadly into structural aberrations and numerical aberrations, and the structural aberrations were further classified into gap (g), chromatid break (ctb), chromatid exchange (cte), chromosome break (csb), chromosome exchange (cse) and others.

For each treatment schedule, Fisher's exact test was applied to the frequency of cells with structural chromosomal aberrations not including gaps (TA), polyploid cells and cells with endoreduplicated chromosomes (significant level: 0.05, one-tailed) to assess the difference between the concurrent negative control group and the test article group. The Cochran-Armitage trend test was not performed because there were no statistical significances in any treatment schedule. When the results fulfilled all the following criteria, the test article is judged to have chromosomal structure abnormality induction (positive): i) the frequency of chromosomal aberration from at least one dose level shows statistically significant increase compared with the concurrent negative control group, ii) this increase is dose-dependent when assessed by the Cochran-Armitage trend test, iii) the increased incidence of chromosomal aberration in the test article group is outside the distribution of the historical negative control data (95% control limits).

In vivo micronucleus test in rat bone marrow

Twenty-eight male Sprague-Dawley [Crl:CD(SD)] rats at 7 weeks of age were purchased from Charles River Laboratories Japan Inc. (Kanagawa, Japan), quarantined and acclimated for 8 days, and 25 healthy rats were used at 8 weeks of age. Animals were housed in an animal room that was controlled to maintain the temperature at 23°C ± 3°C, relative humidity at 50% ± 20%, air ventilation at 10 to 15 times per hour, and 12-hr illumination (7:00 a.m. to 7:00 p.m.). The animals, in groups of two or three, were housed in solid-floored plastic cages (W 440 × D 275 × H 180 mm) with bedding (ConfiNest, Falma Co., Ltd., Tokyo, Japan) with free access to pelleted diet CR-LPF (γ-irradiated, Oriental Yeast Co., Ltd., Tokyo, Japan) and to tap water via water bottles. The animals were assigned to each test group by block randomization using a computer so as to ensure uniformity of group mean body weight.

The oral route was chosen because it is the intended intake route in humans. The dose formulations were administered by gavage using a flexible stomach tube at a dose volume of 10 mL/kg for two consecutive days at an approximately 24-hr interval.

In a previously conducted two-day study in rats (250 to 2000 mg/kg), there were no test article-related changes in the clinical observations or body weights, therefore, the dose levels in the present study were set at 500, 1000 and 2000 mg/kg. In addition, a concurrent negative control group receiving the vehicle (0.1% Tween 80) and a positive control group receiving a single intraperitoneal dose of mitomycin C (MMC, Kyowa Kirin Co., Ltd.) at 2 mg/kg on the day of the second dose of the test article were provided. Each group consisted of 5 male rats.

All animals were observed for survival and clinical signs including any abnormality in the external appearance, nutritional condition, posture, behavior and excrements three times, pre-dose, immediately and approximately 2 hr post-dose, on Days 1 and 2 of the experiment, and body weights were recorded on Days 1, 2 and 3 of the experiment.

On Day 3 of the experiment (approximately 24 hr post-dose of the second dose), animals were euthanized by exsanguination via the abdominal aorta under anesthesia by isoflurane inhalation. The right femur was dissected and both the distal and proximal ends were cut off. Then, the bone marrow cells were flushed out with a small volume of fetal bovine serum (Thermo Fisher Scientific Inc.) into a centrifuge tube. The bone marrow cells were suspended in fetal bovine serum and centrifuged at 1000 rpm for 5 min. Then, the cell pellet was re-suspended in fetal bovine serum and the bone marrow cells were smeared on glass slides (two slides per animal). The smears were air-dried, fixed with methanol for 3 min and air-dried again. For each animal, one slide showing the better smear condition out of two slides were selected, stained with acridine orange and observed using a fluorescent microscope. The frequency of immature erythrocytes (polychromatic erythrocytes [PCEs]) per 500 total erythrocytes (PCEs +

Table 1. Results of reverse mutation test of Monascus Color Y-001 on bacteria [Range-finding test: −S9 mix].

Dose (μg/plate)	Number of revertant colonies per plate [Mean]									
	TA100		TA1535		WP2 *uvrA*		TA98		TA1537	
0	125	105	11	10	23	24	25	27	9	11
	[115]		[11]		[24]		[26]		[10]	
19.5	114	120	9	8	24	16	22	22	8	9
	[117]		[9]		[20]		[22]		[9]	
78.1	120	103	6	7	24	24	24	22	11	8
	[112]		[7]		[24]		[23]		[10]	
313	107	99	6	7	21	19	27	20	12	6
	[103]		[7]		[20]		[24]		[9]	
1250	127	111	5	5	18	20	25	21	4	6
	[119]		[5]		[19]		[23]		[5]	
5000	91*	95*	6*	5*	16*	16*	15*	17*	5*	5*
	[93]		[6]		[16]		[16]		[5]	
Positive control	778	766[a)]	212	222[b)]	99	100[a)]	354	373[c)]	1222	1245[d)]
	[772]		[217]		[100]		[364]		[1234]	

*: Growth inhibition was observed.
a): 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide (0.01 μg/plate)
b): Sodium azide (0.5 μg/plate)
c): 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide (0.1 μg/plate)
d): 2-Methoxy-6-chloro-9-[3-(2-chloroethyl)-aminopropylamino]acridine·2HCl (1.0 μg/plate)

normochromatic erythrocytes [NCEs]) and that of micronucleated immature erythrocytes (micronucleated polychromatic erythrocytes [MNPCEs]) per 4000 PCEs was counted, and their proportions (%) were also calculated.

For the frequency of MNPCEs, pairwise comparison was performed between the negative control group and positive control group, and between the negative control group and each test article group using Fisher's exact test (level of significance: 0.05, one-tailed). The Cochran-Armitage trend test was not performed because there were no statistically significant differences in any test article group.

When the results fulfilled all the following criteria, the test article is judged to be clearly positive: i) in at least one dose level, the frequency of MNPCEs increases compared with the negative control group with statistical significance, ii) this increase is dose-dependent when evaluated with the Cochran-Armitage trend test, iii) the individual frequencies of MNPCEs in the test article group which shows an increase in i) are outside of the mean ± 1.96 SD of the historical negative control data.

RESULTS

Bacterial reverse mutation test (Ames test)

In the range-finding test, growth inhibition was noted at 5000 μg/plate but no increase in the numbers of revertant colonies up to 5000 μg/plate in any of the strains tested, either with or without S9 mix (Tables 1 and 2) was seen. Therefore, the highest dose level in the main test for all tester strains was set at 5000 μg/plate, and 5 lower dose levels were set at 2500, 1250, 625, 313 and 156 μg/plate both with and without metabolic activation in a common ratio of 2.

The results in the main test are presented in Tables 3 and 4.

In the main test, growth inhibition was observed in all tester strains at the two highest doses, 2500 and 5000 μg/plate, both in the presence and absence of S9 mix. There was no increase in the numbers of revertant colonies up to 5000 μg/plate in any of the strains tested, either with or without S9 mix. The negative and positive controls produced acceptable responses, and the acceptance criteria were met and the assay was concluded to be valid.

In vitro chromosomal aberration test

In the range finding test, the relative population doubling (RPD) was less than 50% at ≥ 313 μg/mL in all treatment schedules (Table 5). The 50% cell-growth inhibition concentrations were estimated to be 190 μg/mL in the short-term treatment without S9 mix, 216 μg/mL in the short-term treatment with S9 mix and 166 μg/mL in the continuous treatment (Table 5). Therefore, the dose levels in the main test were set as follows: 1) short-term treatment without metabolic activation: 225, 200, 175,

Table 2. Results of reverse mutation test of Monascus Color Y-001 on bacteria [Range-finding test: +S9 mix].

Dose (μg/plate)	Number of revertant colonies per plate [Mean]									
	TA100		TA1535		WP2 *uvrA*		TA98		TA1537	
0	127	108	12	11	32	30	37	32	9	8
	[118]		[12]		[31]		[35]		[9]	
19.5	127	123	8	8	28	32	31	41	7	8
	[125]		[8]		[30]		[36]		[8]	
78.1	126	110	12	11	36	33	38	42	8	6
	[118]		[12]		[35]		[40]		[7]	
313	97	105	12	8	37	26	30	30	6	10
	[101]		[10]		[32]		[30]		[8]	
1250	124	115	6	6	21	21	23	24	8	7
	[120]		[6]		[21]		[24]		[8]	
5000	95*	95*	6*	6*	16*	18*	21*	25*	7*	4*
	[95]		[6]		[17]		[23]		[6]	
Positive control	999	1007[a)]	233	244[b)]	566	578[c)]	300	311[a)]	99	90[a)]
	[1003]		[239]		[572]		[306]		[95]	

*: Growth inhibition was observed.
a): Benzo[*a*]pyrene (5.0 μg/plate)
b): 2-Aminoanthracene (2.0 μg/plate)
c): 2-Aminoanthracene (10.0 μg/plate)

Table 3. Results of reverse mutation test of Monascus Color Y-001 on bacteria [Main test: −S9 mix].

Dose (μg/plate)	Number of revertant colonies per plate [Mean ± SD]														
	TA100			TA1535			WP2 *uvrA*			TA98			TA1537		
0	110	119	99	10	12	12	23	15	17	22	27	19	10	14	7
	[109 ± 10.0]			[11 ± 1.2]			[18 ± 4.2]			[23 ± 4.0]			[10 ± 3.5]		
156	116	116	115	9	7	7	20	14	24	20	24	27	8	10	8
	[116 ± 0.6]			[8 ± 1.2]			[19 ± 5.0]			[24 ± 3.5]			[9 ± 1.2]		
313	112	118	110	6	10	7	14	21	12	23	17	20	9	13	12
	[113 ± 4.2]			[8 ± 2.1]			[16 ± 4.7]			[20 ± 3.0]			[11 ± 2.1]		
625	108	106	101	6	8	12	15	19	15	27	19	17	7	7	6
	[105 ± 3.6]			[9 ± 3.1]			[16 ± 2.3]			[21 ± 5.3]			[7 ± 0.6]		
1250	90	99	95	5	3	3	15	17	19	19	19	15	7	7	11
	[95 ± 4.5]			[4 ± 1.2]			[17 ± 2.0]			[18 ± 2.3]			[8 ± 2.3]		
2500	99*	94*	87*	4*	4*	2*	16*	16*	15*	16*	18*	18*	9*	9*	3*
	[93 ± 6.0]			[3 ± 1.2]			[16 ± 0.6]			[17 ± 1.2]			[7 ± 3.5]		
5000	88*	90*	88*	4*	3*	3*	16*	15*	14*	16*	17*	15*	3*	4*	4*
	[89 ± 1.2]			[3 ± 0.6]			[15 ± 1.0]			[16 ± 1.0]			[4 ± 0.6]		
Positive control	580	502	552[a)]	243	242	213[b)]	129	140	136[a)]	527	495	525[c)]	1234	1222	1291[d)]
	[545 ± 39.5]			[233 ± 17.0]			[135 ± 5.6]			[516 ± 17.9]			[1249 ± 36.9]		

*: Growth inhibition was observed.
a): 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide (0.01 μg/plate)
b): Sodium azide (0.5 μg/plate)
c): 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide (0.1 μg/plate)
d): 2-Methoxy-6-chloro-9-[3-(2-chloroethyl)-aminopropylamino]acridine·2HCl (1.0 μg/plate)

150, 125, 100 and 75.0 μg/mL, 2) short-term treatment with metabolic activation: 250, 225, 200, 175, 150, 125 and 100 μg/mL, 3) continuous treatment: 200, 175, 150, 125, 100 and 75.0 μg/mL. The results in the main test are presented in Tables 6 to 8.

In the main test, the RPD decreased with increasing dose levels and the RPD was less 50% at the highest dose and each treatment schedule. Therefore, 4 dose

Table 4. Results of reverse mutation test of Monascus Color Y-001 on bacteria [Main test: +S9 mix].

Dose (μg/plate)	Number of revertant colonies per plate [Mean ± SD]														
	TA100			TA1535			WP2 *uvrA*			TA98			TA1537		
0	110	111	101	11	12	13	22	28	26	36	31	31	11	9	11
	[107 ± 5.5]			[12 ± 1.0]			[25 ± 3.1]			[33 ± 2.9]			[10 ± 1.2]		
156	133	111	128	7	9	13	23	29	25	33	28	33	12	7	7
	[124 ± 11.5]			[10 ± 3.1]			[26 ± 3.1]			[31 ± 2.9]			[9 ± 2.9]		
313	109	121	91	9	7	9	27	31	23	35	24	28	12	11	10
	[107 ± 15.1]			[8 ± 1.2]			[27 ± 4.0]			[29 ± 5.6]			[11 ± 1.0]		
625	134	107	92	7	10	11	25	23	25	28	25	21	9	10	7
	[111 ± 21.3]			[9 ± 2.1]			[24 ± 1.2]			[25 ± 3.5]			[9 ± 1.5]		
1250	115	95	116	11	10	7	24	21	27	24	32	24	8	11	6
	[109 ± 11.8]			[9 ± 2.1]			[24 ± 3.0]			[27 ± 4.6]			[8 ± 2.5]		
2500	94*	94*	88*	9*	7*	4*	13*	18*	17*	18*	18*	17*	7*	5*	9*
	[92 ± 3.5]			[7 ± 2.5]			[16 ± 2.6]			[18 ± 0.6]			[7 ± 2.0]		
5000	91*	91*	88*	3*	3*	3*	15*	15*	16*	18*	16*	18*	5*	3*	3*
	[90 ± 1.7]			[3 ± 0.0]			[15 ± 0.6]			[17 ± 1.2]			[4 ± 1.2]		
Positive control	955	997	982[a)]	228	255	225[b)]	561	574	528[c)]	287	271	312[a)]	86	72	96[a)]
	[978 ± 21.3]			[236 ± 16.5]			[554 ± 23.7]			[290 ± 20.7]			[85 ± 12.1]		

*: Growth inhibition was observed.
a): Benzo[*a*]pyrene (5.0 μg/plate)
b): 2-Aminoanthracene (2.0 μg/plate)
c): 2-Aminoanthracene (10.0 μg/plate)

Table 5. Results of chromosomal aberration test of Monascus Color Y-001 on CHL/IU cells [Range-finding test].

Treatment (hr)	6						24		
S9 mix	−			+			−		
Dose (μg/mL)	PD	RPD (%)	Cell-growth inhibition ratio (%)	PD	RPD (%)	Cell-growth inhibition ratio (%)	PD	RPD (%)	Cell-growth inhibition ratio (%)
0	1.30	100	0	1.27	100	0	1.30	100	0
39.1	1.12	86	14	1.23	97	3	1.34	103	-3
78.1	1.23	95	5	1.20	94	6	1.20	92	8
156	0.96	74	26	1.08	85	15	0.77	59	41
313	-0.50	-38	138	-0.09	-7	107	-1.09	-84	184
625	−	−	−	−	−	−	−	−	−
1250	−	−	−	−	−	−	−	−	−
2500	−	−	−	−	−	−	−	−	−
5000	−	−	−	−	−	−	−	−	−
IC_{50} (μg/mL)	190			216			166		

PD: Population doubling = [log (Post-treatment cell number/Initial cell number)] /log 2
RPD: Relative population doubling = PD in the test group/PD in the negative control group × 100
Cell-growth inhibition ratio: 100 − RPD
IC_{50}: Concentration of 50% cell growth inhibition
−: Incalculable due to severe cytotoxicity

levels, the highest dose and 3 lower doses, in each treatment schedule were observed. However, no significant or dose-dependent increases in structural or numerical aberrations were observed at any dose in any of the three treatment schedules. All vehicle control values were within historical negative control ranges and the positive controls induced significant increases in structural aberrations. Thus, all of the acceptance criteria were met and the assay was concluded to be valid.

Table 6. Results of chromosomal aberration test of Monascus Color Y-001 on CHL/IU cells [Main test, Sort-term treatment: −S9 mix].

Treatment (hr)	S9 mix	Dose (μg/mL)	Plate	Number of cells with structural chromosomal aberration[a),b)]								RPD (%)	Plate	Number of cells with numerical chromosomal aberration[b)]			
				Cells[c)] observed	ctb	cte	csb	cse	other	g	Total (−gap)			Cells[d)] observed	Polyploid cell	Endore-duplicated cell	Total
6	−	NC	1	150	1	0	0	0	0	0	1		1	151	1	0	1
		0	2	150	0	1	0	0	0	0	1	100	2	151	1	0	1
			Total	300	1 (0.3)	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.7)		Total	302	2 (0.7)	0 (0.0)	2 (0.7)
			1										1				
		75.0	2					Not observed				89	2			Not observed	
			Total										Total				
			1										1				
		100	2					Not observed				92	2			Not observed	
			Total										Total				
			1										1				
		125	2					Not observed				89	2			Not observed	
			Total										Total				
			1	150	0	1	0	0	0	0	1		1	151	1	0	1
		150	2	150	0	0	0	0	0	0	0	80	2	151	1	0	1
			Total	300	0 (0.0)	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.3)		Total	302	2 (0.7)	0 (0.0)	2 (0.7)
			1	150	0	1	0	0	0	0	1		1	151	1	0	1
		175	2	150	0	1	0	0	0	0	1	60	2	151	1	0	1
			Total	300	0 (0.0)	2 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.7)		Total	302	2 (0.7)	0 (0.0)	2 (0.7)
			1	150	0	0	0	0	0	0	0		1	152	2	0	2
		200	2	150	0	1	0	0	0	0	1	55	2	151	1	0	1
			Total	300	0 (0.0)	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.3)		Total	303	3 (1.0)	0 (0.0)	3 (1.0)
			1	150	1	2	0	0	0	0	2		1	150	0	0	0
		225	2	150	1	1	0	0	0	0	2	43	2	153	3	0	3
			Total	300	2 (0.7)	3 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (1.3)		Total	303	3 (1.0)	0 (0.0)	3 (1.0)
		PC	1	150	4	23	0	0	0	1	26		1	150	0	0	0
		0.075	2	150	5	21	0	0	0	0	26	63	2	150	0	0	0
			Total	300	9 (3.0)	44 (14.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.3)	52* (17.3)		Total	300	0 (0.0)	0 (0.0)	0 (0.0)

a): ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange, other: including fragmentation, g: chromatid or chromosome gap
b): Value in the parentheses indicates percentage against the total number of cells observed. c): Diploid cells d): Diploid, polyploid, and aneuploid cells
NC: Negative control (water for injection), PC: Positive control (mitomycin C)
RPD: Relative population doubling
*: $p < 0.05$ (significantly different between the negative and positive controls by Fisher's exact test)

Table 7. Results of chromosomal aberration test of Monascus Color Y-001 on CHL/IU cells [Main test, Sort-term treatment: +S9 mix].

Treatment (hr)	S9 mix	Dose (μg/mL)	Plate	Number of cells with structural chromosomal aberration[a),b)]								RPD (%)	Plate	Number of cells with numerical chromosomal aberration[b)]			
				Cells[c)] observed	ctb	cte	csb	cse	other	g	Total (−gap)			Cells[d)] observed	Polyploid cell	Endore-duplicated cell	Total
		NC	1	150	1	0	0	0	0	0	1		1	151	1	0	1
		0	2	150	1	1	0	0	0	0	2	100	2	151	1	0	1
			Total	300	2 (0.7)	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (1.0)		Total	302	2 (0.7)	0 (0.0)	2 (0.7)
			1										1				
		100	2					Not observed				89	2			Not observed	
			Total										Total				
			1										1				
		125	2					Not observed				89	2			Not observed	
			Total										Total				
			1										1				
		150	2					Not observed				89	2			Not observed	
			Total										Total				
			1	150	0	0	0	0	0	0	0		1	152	1	1	2
6	+	175	2	150	0	1	0	0	0	0	1	83	2	151	1	0	1
			Total	300	0 (0.0)	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.3)		Total	303	2 (0.7)	1 (0.3)	3 (1.0)
			1	150	0	0	0	0	0	0	0		1	153	3	0	3
		200	2	150	0	1	0	0	0	0	1	74	2	150	0	0	0
			Total	300	0 (0.0)	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.3)		Total	303	3 (1.0)	0 (0.0)	3 (1.0)
			1	150	1	0	0	0	0	0	1		1	151	0	1	1
		225	2	150	0	1	0	0	0	0	1	55	2	152	1	1	2
			Total	300	1 (0.3)	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.7)		Total	303	1 (0.3)	2 (0.7)	3 (1.0)
			1	150	0	1	0	0	0	0	1		1	151	1	0	1
		250	2	150	0	1	0	0	0	0	1	47	2	152	1	1	2
			Total	300	0 (0.0)	2 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.7)		Total	303	2 (0.7)	1 (0.3)	3 (1.0)
		PC	1	150	6	97	0	0	0	0	98		1	150	0	0	0
		14	2	150	6	96	0	0	0	0	99	51	2	150	0	0	0
			Total	300	12 (4.0)	193 (64.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	197* (65.7)		Total	300	0 (0.0)	0 (0.0)	0 (0.0)

a): ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange, other: including fragmentation, g: chromatid or chromosome gap
b): Value in the parentheses indicates percentage against the total number of cells observed. c): Diploid cells d): Diploid, polyploid, and aneuploid cells
NC: Negative control (water for injection), PC: Positive control (cyclophosphamide monohydrate)
RPD: Relative population doubling
*: $p < 0.05$ (significantly different between the negative and positive controls by Fisher's exact test)

Table 8. Results of chromosomal aberration test of Monascus Color Y-001 on CHL/IU cells [Main test, Continuous treatment: 24 hr].

Treatment (hr)	S9 mix	Dose (μg/mL)	Plate	Number of cells with structural chromosomal aberration[a),b)]								RPD (%)	Plate	Number of cells with numerical chromosomal aberration[b)]			
				Cells[c)] observed	ctb	cte	csb	cse	other	g	Total (−gap)			Cells[d)] observed	Polyploid cell	Endore-duplicated cell	Total
24	–	NC	1	150	0	1	0	0	0	0	1		1	152	2	0	2
		0	2	150	1	0	0	0	0	0	1	100	2	151	1	0	1
			Total	300	1 (0.3)	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.7)		Total	303	3 (1.0)	0 (0.0)	3 (1.0)
		75.0	1										1				
			2					Not observed				88	2			Not observed	
			Total										Total				
		100	1										1				
			2					Not observed				88	2			Not observed	
			Total										Total				
		125	1	150	0	0	0	0	0	0	0		1	151	1	0	1
			2	150	0	1	0	0	0	0	1	81	2	152	2	0	2
			Total	300	0 (0.0)	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.3)		Total	303	3 (1.0)	0 (0.0)	3 (1.0)
		150	1	150	1	0	0	0	0	0	1		1	151	1	0	1
			2	150	1	0	0	0	0	0	1	63	2	151	1	0	1
			Total	300	2 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.7)		Total	302	2 (0.7)	0 (0.0)	2 (0.7)
		175	1	150	0	1	0	0	0	0	1		1	150	0	0	0
			2	150	0	0	0	0	0	0	0	58	2	151	1	0	1
			Total	300	0 (0.0)	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.3)		Total	301	1 (0.3)	0 (0.0)	1 (0.3)
		200	1	150	0	0	0	0	0	0	0		1	150	0	0	0
			2	150	0	1	0	0	0	0	1	45	2	150	0	0	0
			Total	300	0 (0.0)	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.3)		Total	300	0 (0.0)	0 (0.0)	0 (0.0)
		PC	1	150	4	26	0	0	0	0	28		1	150	0	0	0
		0.050	2	150	6	28	0	0	0	0	33	78	2	150	0	0	0
			Total	300	10 (3.3)	54 (18.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	61* (20.3)		Total	300	0 (0.0)	0 (0.0)	0 (0.0)

a): ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange, other: including fragmentation, g: chromatid or chromosome gap
b): Value in the parentheses indicates percentage against the total number of cells observed. c): Diploid cells d): Diploid, polyploid, and aneuploid cells
NC: Negative control (water for injection), PC: Positive control (mitomycin C)
RPD: Relative population doubling
*: $p < 0.05$ (significantly different between the negative and positive controls by Fisher's exact test)

Table 9. Results of *in vivo* bone marrow micronucleus test for Monascus Color Y-001 in rats.

Group	Dose (mg/kg)		No. of MNPCE in 4000 PCE		No. of PCE in 500 erythrocytes	
			Frequency	Incidence (%)	Frequency	Incidence (%)
Negative control	0	N	5	5	5	5
		Mean ± SD	7 ± 1	0.17 ± 0.03	306 ± 39	61.2 ± 7.7
		Min/Max	5/8	0.13/0.20	255/347	51.0/69.4
Low	500	N	5	5	5	5
		Mean ± SD	5 ± 2	0.13 ± 0.05	242 ± 38	48.4 ± 7.5#
		Min/Max	2/7	0.05/0.18	184/279	36.8/55.8
Middle	1000	N	5	5	5	5
		Mean ± SD	5 ± 2	0.13 ± 0.04	232 ± 9	46.4 ± 1.9#
		Min/Max	3/7	0.08/0.18	218/242	43.6/48.4
High	2000	N	5	5	5	5
		Mean ± SD	5 ± 2	0.12 ± 0.04	200 ± 54	40.0 ± 10.9##
		Min/Max	3/7	0.08/0.18	118/268	23.6/53.6
Positive control[a)]	2[a)]	N	5	5	5	5
		Mean ± SD	124 ± 22*	3.10 ± 0.55	215 ± 32	43.0 ± 6.4
		Min/Max	97/154	2.43/3.85	167/252	33.4/50.4

MNPCE: Micronucleated polychromatic erythrocytes, PCE: Polychromatic erythrocytes
a): Single intraperitoneal injection of Mitomycin C
*: $P < 0.05$ (Significantly different from the negative control group by Fisher's exact test)
#: $P < 0.05$, ##: $P < 0.01$ (Significantly different from the negative control group by Dunnett's test)

In vivo micronucleus test in rat bone marrow

The results are presented in Table 9.

There were no deaths or test article-related changes in the clinical signs at any of the doses. A body weight loss was noted at 2000 mg/kg on Day 3 of the experiment. Dose-related decreases in the polychromatic erythrocytes (PCE) were observed in the test article groups, suggesting systemic exposure to Monascus Color Y-001. No significant difference in the frequency of MNPEC was observed between each test article group and the negative control group. The incidences of MNPEC in the negative and positive controls were similar to those in the historical control data, confirming the validity of the present study.

DISCUSSION

The genotoxic potential of Monascus Color Y-001 was assessed in the standard battery of genotoxicity assays: the *in vitro* reverse mutation test in bacteria (Ames test), the *in vitro* chromosomal aberration assay in mammalian cells and the *in vivo* micronucleus assay in rats.

Monascus Color Y-001 was clearly negative in these mutagenicity and genotoxicity assays. In conclusion, Monascus Color Y-001 does not present any genotoxic risk in humans.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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