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Original Article

A 90-day oral repeated-dose toxicity study of Monascus Color Y-001 in rats

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ABSTRACT — Monascus Color Y-001, a natural food dye produced from *Monascus purpureus* fermentation, was administered orally by gavage to male and female SD rats for 90 days at doses of 0 (vehicle: 0.1% Tween 80, 10 mL/kg bw), 100, 300 and 1000 mg/kg/day. During the treatment period, there was no death, and test article effects on clinical signs were limited to reddish feces, soiled perineal region (reddish color) and salivation that were observed in both sexes at 300/1000 mg/kg/day. Prolongation in PT and APTT occurred in males at 1000 mg/kg/day, and the changes were without any evidence suggesting hemorrhage and/or hepatic dysfunction. Treatment-related histopathological findings were noted in thymus, liver and kidney, and were limited to the females at 1000 mg/kg/day. These findings included decreased cellularity in thymus with decreased thymus weights attributed to nonspecific stress, centrilobular hepatocellular hypertrophy with increase of liver weights attributed to adaptive change, and vacuolation of proximal tubules in kidneys accompanied with related parameter changes in urinalysis. From these results, the no-observed-adverse-effect level (NOAEL) was judged to be 300 mg/kg/day both in male and female rats.

Key words: Monascus Color Y-001, 90-day toxicity study, Rat

INTRODUCTION

Monascus Color Y-001, one of the Monascus colors produced from *Monascus purpureus* 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 as part of the safety assessment of this colorant as a food additive. Recently, genotoxicity study results have been reported and concluded that Monascus Color Y-001 does not possess any genotoxic risk in humans (Sato *et al*., 2021). In the current paper, we report the results of a 90-day oral repeated-dose toxicity study of Monascus Color Y-001.

MATERIALS AND METHODS

The study was conducted at DIMS Institute of Medical Science, Inc., in compliance with Good Laboratory Practice (GLP) regulations and in accordance with the Redbook 2000 guidelines (FDA). The present study was conducted in accordance with the "Act on Welfare and Management of Animals" (Low No. 39, June 2019), "Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain" (Notice No. 84 of the Ministry of Environment dated September, 2013), "Guidelines for Proper Conduct of Animal Experiments" (Science Council of Japan, June, 2006), "Basic policies for the conduct of animal experiment in academic research institutions" (Notice No. 02201, Ministry of Health, Labour and Welfare, February 2015 and Notice

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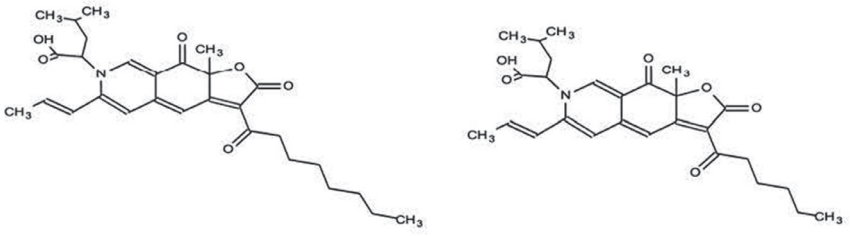

N-leucyl monascorubrin N-leucyl rubropunctatin

Fig. 1. Chemical structure of N-leucyl monascorubrin and N-leucyl rubropunctatin, main components of Monascus Color Y-001.

No. 18 Nou-Kai 307, Ministry of Agriculture, Forestry and Fisheries, June 2006), and "Standards for Care and Use of Laboratory Animals at DIMS Institute of Medical Science, Inc." (August 11, 2020). This study was approved by the animal experiment committee of the DIMS Institute of Medical Science, Inc.

Test article

Monascus Color Y-001, provided by YAEGAKI Bio-industry, Inc. (Hyogo, Japan), is dark red powder having a unique odor. Monascus Color Y-001 is composed of two main components, N-leucyl monascorubrin and N-leucyl rubropunctatin (Fig. 1). Additional components accounted for less than 10% of the total by weight in all 4 batches used in this study. Polyoxyethylene sorbitan monooleate (Tween 80) (Nacalai Tesque, Inc., Kyoto, Japan) was dissolved in distilled water at concentration of 0.1% (w/v), and this solution (0.1% Tween 80) was used as vehicle. Monascus Color Y-001 was suspended in 0.1% Tween 80 for each dose level preparation.

Animals

Five-week-old male and female Crl:CD(SD) rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan), and acclimated for 7 days before allocation. Two rats were housed in respective clear polypropylene cages (W 257 × D 426 × H 200 mm) with soft wood chip bedding (Japan SLC, Inc., Shizuoka, Japan) in an animal facility with a temperature of 22 ± 3ºC, humidity of 55 ± 15%, ventilation frequency of at least 10 times/hr, and a 12-hr light/dark cycle (7:00 AM -7:00 PM). MF pellet diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and Ichinomiya City tap water were available *ad libitum*. Rats were allocated based on randomized body weights into 4 groups in each sex (10 rats/group/sex), and administration of the test article was started at 6 weeks of age.

Study design

Based on the results of a 14-day dose-finding study (Dose levels: 800, 1000 and 1200 mg/kg/day), the dose levels were set at 0 (vehicle control), 100, 300 and 1000 mg/kg/day in the study. The study design is shown in Table 1. The animals were treated orally by gavage once daily for 90 days, and the dosing volume (10 mL/kg bw) was adjusted to the latest body weight for individual rats.

Observations and examinations

All animals were checked twice daily for general conditions. Functional observation battery (FOB) was achieved through weekly detailed clinical observations (home cage and in a standard field), a function test (sensory reactivity to different stimuli, and grip strength) and locomotor activity during the final week (Week 13). Body weights and two-day food and water consumption were measured weekly using an electric balance. Ophthalmo-

Table 1. Dosage group design.

Test article	Dose (mg/kg/day)	Dose volume (mL/kg bw)	Concentration (mg/mL)	Number of rats	
				Male	Female
Control (vehicle)[a]	0	10	0	10	10
Monascus Color Y-001	100	10	10	10	10
	300	10	30	10	10
	1000	10	100	10	10

a: 0.1% Tween 80 in distilled water.

scopic examination was performed before treatment and during the final week. On Week 13, 10 animals/sex/group were placed in urine-collection cages with food and water, and 4-hr and 20-hr urine outputs were collected. Urine color and sediments, and some parameters using a test paper (Multistix, Siemens Healthcare Diagnostics K.K., Tokyo, Japan) were examined using 4-hr urine. Urine volume, specific gravity and urine electrolytes were measured with 20-hr urine. After the treatment period, animals were deprived of food overnight, and blood samples were collected from the abdominal aorta under isoflurane anesthesia. For hematology, blood samples were analyzed using an automated hematology analyzer, model XT-2000i (Sysmex Co., Hyogo, Japan), and Automated Blood Coagulation Analyzer CA-530 (Sysmex Co.). For clinical biochemistry, serum samples were obtained by centrifuging blood samples, and evaluation was performed using an automatic analyzer model 3500 (Hitachi, Ltd., Tokyo, Japan).

Postmortem examinations

All surviving animals were euthanized by bleeding from abdominal aorta under isoflurane anesthesia, and subjected to necropsy. Heart, spleen, lymph node (mandibular, mesentery), thymus, pituitary gland, thyroid, adrenal gland, nasal cavity, trachea, lung (including bronchi), salivary gland (submandibular gland, sublingual gland), esophagus, stomach, small intestine (duodenum, jejunum, ileum), large intestine (cecum, colon, rectum), liver, pancreas, kidney, urinary bladder, testis, prostate, seminal vesicle, epididymis, ovary, uterus, mammary gland, vagina, brain, spinal cord, sciatic nerve, aorta, eyeball, Harderian gland, skin, bone and bone marrow (femur, sternum), skeletal muscle, Zymbal's gland and other macroscopic lesion sites were excised, fixed in 10% buffered formalin solution and processed for histopathological examination. Brain, heart, lung, liver, kidney, spleen, thymus, pituitary gland, thyroid, adrenal gland, salivary gland, testis, epididymis, prostate, seminal vesicle, ovary and uterus were weighed using an electronic balance (Type CP323S and BP61S, Sartorius Japan K.K., Tokyo, Japan). Histopathological examination was performed on the above organs and tissues from the control and high-dose groups. Kidney, liver and stomach in the low- and middle-dose groups were additionally subjected to histopathological examination.

Statistical analysis

Significant differences between the control and test article groups were analyzed and evaluated at $p < 0.05$ or $p < 0.01$. The numerical data were assessed using Bartlett's test. When homogeneous in the Bartlett's test, the data were analyzed using Dunnett's multiple comparison test (two-sided); when not, they were analyzed with Steel's test (two-sided). Categorical urinalysis data with grade were firstly analyzed by the chi-square test using m x n contingency table (two-sided, m: dose level, n: number of grade). When the p-value showed significance, a 2 x n contingency table was sequentially used for the chi-square test to compare control group with each dose group. Analysis of incidence data were performed using the Fisher's exact probability test (one-sided). The grade data were analyzed using the Wilcoxon's rank-sum test (two-sided). Statistical analysis was not performed for the data of observation and qualitative data of functional observations.

RESULTS

Observations and examinations

There was no death at any dose in the study during the treatment period. Reddish feces were observed at 300 mg/kg/day and higher through the treatment period. At 1000 mg/kg/day, salivation and soiled perineal region (reddish in color) were also observed. These findings were related to the properties of the test article, a powder with a unique odor and red color, and were of little toxicological significance.

In the FOB, a statistically significant decrease of forelimb grip strength was noted in males at doses of 300 mg/kg/day and higher (data not shown). The change was limited in males and not observed in hindlimb grip strength, and not observed in females at any dose. In addition, the measurement of grip strength was performed only one time (Week 13) during the treatment period. Therefore, this change is unlikely to be related to administration of the test article.

Body weight, food and water consumption were shown in Table 2. At 1000 mg/kg/day, mean body weight showed a slight downward trend in males and females during the treatment period. Significant increases in food consumption in males and water consumption in both sexes were observed at 1000 mg/kg/day.

No treatment-related changes were observed in ophthalmoscopic examination performed on pre-treatment and Week 13 (data not shown).

In urinalysis, urine color change in urine, pale to deep yellow, was observed both in males and females at 1000 mg/kg/day (data not shown). The change was attributed to the color of the test article and/or its metabolite in urine. Increase in ketone bodies, glucose, protein, urobilinogen and bilirubin in males at 1000 mg/kg/day, and

Table 2. Body weights, food consumption and water consumption data on the first week (Week 1) and the final week (Week 13) of rats administered Monascus Color Y-001 for 90-day.

	Dose (mg/kg/day)			
	Control	100	300	1000
[male]				
Body weights (g)				
Week 1	209.7 ± 8.2	209.9 ± 6.9	209.2 ± 7.8	209.0 ± 5.0
Week 13	539.1 ± 51.1	565.2 ± 49.2	553.2 ± 65.7	486.6 ± 23.8
Food consumption (g/animal/day)				
Week 1	23.8 ± 1.1	23.9 ± 1.6	24.1 ± 1.2	23.4 ± 1.6
Week 13	24.2 ± 1.9	25.9 ± 2.6	26.1 ± 1.7	27.2 ± 2.0**
Water consumption (g/animal/day)				
Week 1	31.8 ± 1.6	33.4 ± 2.0	31.2 ± 2.5	42.7 ± 1.7**
Week 13	37.3 ± 8.5	37.6 ± 8.7	37.3 ± 5.2	57.7 ± 5.5**
[female]				
Body weights (g)				
Week 1	158.5 ± 4.8	159.8 ± 6.2	159.6 ± 6.1	159.4 ± 4.4
Week 13	321.1 ± 22.9	309.6 ± 28.1	310.1 ± 23.2	297.6 ± 18.6
Food consumption (g/animal/day)				
Week 1	17.2 ± 0.7	16.4 ± 0.6#	15.8 ± 1.0##	16.2 ± 1.5
Week 13	16.8 ± 1.0	17.3 ± 1.7	16.8 ± 1.3	17.3 ± 1.4
Water consumption (g/animal/day)				
Week 1	23.9 ± 3.5	24.3 ± 1.5	23.8 ± 2.6	31.1 ± 2.8**
Week 13	26.3 ± 5.1	29.9 ± 9.0	25.4 ± 2.9	45.6 ± 9.1##

Data are presented as mean ± SD.
**: Significantly different from the control group at $p < 0.01$ (Dunnett test).
#, ##: Significantly different from the control group at $p < 0.05$, 0.01 (Steel test), respectively.

decrease in electrolytes and specific gravity and increase in urine volume at the same dose in females were noted (Tables 3 and 4).

In hematological examination, prothrombin time (PT) and activated partial thromboplastin time (APTT) were significantly prolonged in males at 1000 mg/kg/day (Table 5). In females at 1000 mg/kg/day, significant prolongation of PT was also noted, but the change was negligible since the degree of the change was very slight. There were no toxicological changes in erythrocyte and leukocyte parameters at any dose.

In clinical biochemistry, statistically significant increases in blood urea nitrogen and decreases in glucose were observed in both males and females at 1000 mg/kg/day. In addition to these changes, significant increases in alanine aminotransferase (ALT), creatinine, inorganic phosphate, and a decrease in Na concentration were found in males, and significant increases in alkaline phosphatase, total bilirubin, total bile acid, triglyceride, and a decrease in Cl concentration were found in females (Table 6). The other findings and variations in parameters were comparable to those observed in untreated rats in this laboratory.

Postmortem examinations

In necropsy findings, dark red contents in the gastrointestinal tracts at 100 mg/kg/day and higher, and red colored fur in anogenital region at 1000 mg/kg/day were observed both in males and females. These findings were attributed to the color of the test article, and the findings possessed no toxicologic significance.

In organ weights, liver weights were increased, and thymus weights were decreased only in females at 1000 mg/kg/day (Tables 7 and 8).

In histopathological examination, treatment-related changes were only observed in thymus, liver and kidneys in females at 1000 mg/kg/day and were not found in males (Table 9). Decreased cellularity of lymphocytes was noted in cortex in thymus. Hepatocellular hypertrophy had centrilobular distribution and showed ground glass appearance in the liver. Proximal tubular vacuolation was characterized by small to medium-sized clear vacuoles localized in the basal part of the proximal tubules in the kidney.

Table 3. Urine qualitative data of rats administered Monascus Color Y-001 for 90-day.

Dose (mg/kg/day)	pH						Occult blood				Ketone bodies (mg/dL)				
	6.5	7.0	7.5	8.0	8.5	9.0	-	±	1+	2+	-	5	15	40	
[male]															
Control	0	1	0	2	6	1	7	3	0	0	4	5	1	0	
100	0	0	2	1	5	2	5	4	1	0	8	2	0	0	
300	1	1	1	2	4	1	5	5	0	0	4	3	3	0	
1000	1	3	2	2	2	0	8	2	0	0	0	1	8	1	**
[female]															
Control	0	1	2	2	5	0	10	0	0	0	10	0	0	0	
100	0	1	0	1	7	1	7	2	0	1	9	1	0	0	
300	0	0	2	1	6	1	9	1	0	0	10	0	0	0	
1000	1	1	0	2	3	3	10	0	0	0	7	3	0	0	

Table 3. (Continued).

Dose (mg/kg/day)	Glucose (mg/dL)		Protein (mg/dL)				Urobilinogen (EU/dL)			Bilirubin		
	-	100	-	±	30	100	0.1	1		-	1+	2+
[male]												
Control	10	0	6	4	0	0	10	0		10	0	0
100	10	0	5	4	1	0	10	0		10	0	0
300	10	0	3	3	3	1	10	0		9	1	0
1000	7	3	0	1	3	6	** 5	5	**	6	3	1
[female]												
Control	10	0	8	1	1	0	10	0		10	0	0
100	10	0	7	2	1	0	9	1		10	0	0
300	10	0	5	4	1	0	10	0		10	0	0
1000	9	1	6	2	2	0	8	2		10	0	0

Values are number of animals with findings.
Symbols: -; negative, ±; very slight, 1+; slight, 2+; moderate.
**: Significantly different from the control group at $p < 0.01$ [Chi-square test (mXn)].

Table 4. Urine chemistry data of rats administered Monascus Color Y-001 for 90-day.

	Dose (mg/kg/day)			
	Control	100	300	1000
[male]				
Na (mmol/L)	31.8 ± 25.9	38.3 ± 43.1	60.5 ± 49.4	32.0 ± 17.6 (9)
K (mmol/L)	162.89 ± 62.78	158.05 ± 73.80	227.61 ± 98.07	97.97 ± 86.36
Cl (mmol/L)	44.5 ± 37.0	40.0 ± 40.2	102.2 ± 62.1* (9)	49.3 ± 47.0
Specific gravity	1.042 ± 0.012	1.039 ± 0.018	1.051 ± 0.018	1.044 ± 0.020
Urine volume (g)	12.8 ± 4.5	16.1 ± 6.4	14.0 ± 4.8	14.7 ± 7.3
[female]				
Na (mmol/L)	76.6 ± 27.4	82.1 ± 44.1	73.5 ± 24.6	33.2 ± 19.5**
K (mmol/L)	231.02 ± 73.59	222.70 ± 78.35	217.26 ± 88.94	105.94 ± 68.42**
Cl (mmol/L)	105.8 ± 45.2	112.4 ± 51.2	100.6 ± 49.5	56.5 ± 36.0
Specific gravity	1.050 ± 0.018	1.048 ± 0.016	1.052 ± 0.013	1.027 ± 0.015**
Urine volume (g)	11.8 ± 6.1	11.6 ± 6.4	8.4 ± 3.0	20.9 ± 14.3

Data are presented as mean ± SD.
Number in parentheses indicates the number of animals examined since the values under the measuring limit were omitted.
*, **: Significantly different from the control group at $p < 0.05$, 0.01 (Dunnett test), respectively.

Table 5. Hematology data of rats administered Monascus Color Y-001 for 90-day.

	Dose (mg/kg/day)			
	Control	100	300	1000
[male]				
Red blood cell counts ($\times 10^4$/μL)	874 ± 44	852 ± 40	849 ± 50	879 ± 26
Hematocrit (%)	42.0 ± 1.5	42.0 ± 2.3	41.4 ± 1.9	43.1 ± 2.1
Hemoglobin (g/dL)	15.5 ± 0.6	15.5 ± 0.7	15.3 ± 0.8	15.8 ± 0.7
MCV (fL)	48.1 ± 2.2	49.3 ± 1.8	48.8 ± 1.7	49.0 ± 1.5
MCH (pg)	17.8 ± 0.6	18.1 ± 0.5	18.0 ± 0.6	18.0 ± 0.5
MCHC (g/dL)	36.9 ± 0.8	36.8 ± 0.5	36.9 ± 0.3	36.8 ± 0.6
Platelet counts ($\times 10^4$/μL)	111.6 ± 9.9	112.1 ± 11.0	108.4 ± 9.2	112.1 ± 8.0
Reticulocyte counts ($\times 10^4$/μL)	27.7 ± 5.1	30.0 ± 5.7	25.9 ± 4.6	26.2 ± 7.5
White blood cell counts ($\times 10^2$/μL)	85.1 ± 21.6	78.7 ± 6.9	91.8 ± 30.9	73.8 ± 23.6
Lymphocyte counts ($\times 10^2$/μL)	63.0 ± 16.9	60.0 ± 7.4	69.0 ± 24.2	54.2 ± 20.4
Neutrophil counts ($\times 10^2$/μL)	16.8 ± 8.5	14.0 ± 3.9	17.6 ± 6.5	15.0 ± 5.7
Eosinophil counts ($\times 10^2$/μL)	1.5 ± 0.5	1.5 ± 0.5	1.4 ± 0.8	1.2 ± 0.5
Basophil counts ($\times 10^2$/μL)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Monocyte counts ($\times 10^2$/μL)	3.8 ± 1.5	3.3 ± 0.7	3.8 ± 1.4	3.3 ± 1.4
PT (sec)	14.8 ± 4.1	16.3 ± 5.9	15.4 ± 4.6	22.1 ± 5.4**
APTT (sec)	18.5 ± 3.6	18.8 ± 2.5	18.2 ± 3.7	25.9 ± 4.3**
[female]				
Red blood cell counts ($\times 10^4$/μL)	781 ± 27	765 ± 37	756 ± 44	775 ± 63
Hematocrit (%)	40.7 ± 1.8	40.1 ± 1.2	39.2 ± 1.8	38.9 ± 1.8
Hemoglobin (g/dL)	15.0 ± 0.7	14.8 ± 0.5	14.4 ± 0.7	14.5 ± 0.9
MCV (fL)	52.2 ± 2.6	52.6 ± 1.5	51.9 ± 1.6	50.4 ± 3.0
MCH (pg)	19.2 ± 0.8	19.4 ± 0.4	19.0 ± 0.5	18.8 ± 0.7
MCHC (g/dL)	36.9 ± 0.7	36.8 ± 0.6	36.7 ± 0.4	37.3 ± 1.1
Platelet counts ($\times 10^4$/μL)	114.5 ± 10.1	111.7 ± 14.4	116.9 ± 17.9	101.0 ± 23.3
Reticulocyte counts ($\times 10^4$/μL)	22.5 ± 4.8	21.3 ± 6.2	24.1 ± 4.6	25.1 ± 7.6
White blood cell counts ($\times 10^2$/μL)	34.0 ± 9.8	39.5 ± 14.4	49.0 ± 20.0	34.4 ± 10.8
Lymphocyte counts ($\times 10^2$/μL)	25.9 ± 7.5	30.9 ± 11.5	39.7 ± 17.0*	22.0 ± 8.6
Neutrophil counts ($\times 10^2$/μL)	5.9 ± 3.4	6.4 ± 3.0	7.1 ± 4.2	10.1 ± 6.7
Eosinophil counts ($\times 10^2$/μL)	0.8 ± 0.4	0.8 ± 0.4	0.7 ± 0.4	0.4 ± 0.3
Basophil counts ($\times 10^2$/μL)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Monocyte counts ($\times 10^2$/μL)	1.4 ± 0.5	1.5 ± 0.6	1.4 ± 0.5	2.0 ± 0.9
PT (sec)	9.9 ± 0.4	9.9 ± 0.3	9.9 ± 0.3	10.7 ± 1.1# (9)
APTT (sec)	12.9 ± 0.6	13.8 ± 1.8	13.5 ± 1.1	12.2 ± 1.1

Data are presented as mean ± SD.

MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PT: prothrombin time; APTT: activated partial thromboplastin time.

Number in parentheses indicates the number of animals examined since the poor state samples were omitted.

*, **: Significantly different from the control group at $p < 0.05$, 0.01 (Dunnett test), respectively.

#: Significantly different from the control group at $p < 0.05$ (Steel test).

DISCUSSION

Monascus Color Y-001 was administered orally by gavage to male and female rats at doses of 0 (vehicle), 100, 300 and 1000 mg/kg/day for 90 days to assess nonclinical safety.

No death was observed in the study, and the dose of 1000 mg/kg/day of Monascus Color Y-001 was well tolerated in rats during the 13-week treatment period.

Prolongation in PT and APTT occurred in males at 1000 mg/kg/day. PT and APTT prolongation can be caused by various factors such as deficiency, dysfunction or inhibition of coagulation factors; vitamin K deficiency; or vitamin K antagonists (Winter *et al*., 2017; Tefferi

Table 6. Clinical biochemistry data of rats administered Monascus Color Y-001 for 90-day.

	Dose (mg/kg/day)			
	Control	100	300	1000
[male]				
AST (U/L)	102 ± 28	102 ± 23	106 ± 19	91 ± 20
ALT (U/L)	35 ± 8	32 ± 7	32 ± 7	49 ± 6**
Alkaline phosphatase (U/L)	330 ± 94	310 ± 80	270 ± 67	262 ± 54
γ-GTP (U/L)	0.5 ± 0.1 (8)	0.5 ± 0.1 (9)	0.5 ± 0.1	0.5 ± 0.1
Total bilirubin (mg/dL)	0.03 ± 0.01	0.03 ± 0.01 (5)	0.03 ± 0.02 (5)	0.05 ± 0.02
Total bile acid (μmol/L)	28.2 ± 16.8	30.6 ± 19.3	11.1 ± 4.2#	7.0 ± 3.6##
Blood urea nitrogen (mg/dL)	14.3 ± 1.9	14.1 ± 1.8	13.6 ± 1.3	16.7 ± 2.2*
Creatinine (mg/dL)	0.25 ± 0.03	0.27 ± 0.03	0.26 ± 0.03	0.32 ± 0.03**
Glucose (mg/dL)	150 ± 17	153 ± 20	150 ± 19	122 ± 16**
Total cholesterol (mg/dL)	47 ± 13	49 ± 9	50 ± 8	51 ± 8
Phospholipid (mg/dL)	84 ± 16	87 ± 11	90 ± 13	87 ± 10
Triglyceride (mg/dL)	48 ± 19	56 ± 15	69 ± 58	32 ± 9
Total protein (g/dL)	5.9 ± 0.1	6.1 ± 0.2	5.8 ± 0.3	5.8 ± 0.2
Albumin (g/dL)	4.0 ± 0.2	4.0 ± 0.1	3.9 ± 0.2	4.0 ± 0.2
A/G ratio	2.18 ± 0.33	1.93 ± 0.15	2.00 ± 0.16	2.28 ± 0.37
Inorganic phosphate (mg/dL)	6.7 ± 0.4	6.8 ± 0.5	6.8 ± 0.7	7.5 ± 0.3**
Ca (mg/dL)	10.2 ± 0.3	10.2 ± 0.2	10.1 ± 0.2	10.0 ± 0.3
Mg (mg/dL)	2.2 ± 0.2	2.2 ± 0.1	2.1 ± 0.1	2.4 ± 0.1
Na (mmol/L)	143.0 ± 1.6	142.8 ± 1.4	142.8 ± 1.4	140.1 ± 1.8**
K (mmol/L)	4.53 ± 0.31	4.73 ± 0.31	4.55 ± 0.20	4.46 ± 0.24
Cl (mmol/L)	104.6 ± 1.4	103.6 ± 1.3	104.7 ± 0.9	103.8 ± 1.0
[female]				
AST (U/L)	93 ± 14	87 ± 20	89 ± 18	100 ± 25
ALT (U/L)	31 ± 10	29 ± 5	27 ± 6	36 ± 9
Alkaline phosphatase (U/L)	138 ± 26	155 ± 41	134 ± 30	825 ± 757##
γ-GTP (U/L)	0.6 ± 0.2 (9)	0.7 ± 0.2 (9)	0.5 ± 0.2 (7)	0.6 ± 0.2 [9]
Total bilirubin (mg/dL)	0.04 ± 0.01	0.05 ± 0.02 (9)	0.06 ± 0.02 (9)	0.08 ± 0.03**
Total bile acid (μmol/L)	14.0 ± 6.5	17.3 ± 8.8	17.1 ± 6.2	47.6 ± 31.4#
Blood urea nitrogen (mg/dL)	13.0 ± 1.5	13.2 ± 1.8	15.0 ± 2.1	19.6 ± 3.3**
Creatinine (mg/dL)	0.30 ± 0.04	0.30 ± 0.03	0.33 ± 0.05	0.32 ± 0.04
Glucose (mg/dL)	136 ± 10	151 ± 22	156 ± 29	111 ± 24#
Total cholesterol (mg/dL)	68 ± 11	69 ± 14	60 ± 14	67 ± 13
Phospholipid (mg/dL)	130 ± 13	132 ± 18	123 ± 15	129 ± 20
Triglyceride (mg/dL)	24 ± 10	26 ± 10	37 ± 16	186 ± 215##
Total protein (g/dL)	6.4 ± 0.4	6.5 ± 0.3	6.5 ± 0.3	6.3 ± 0.4
Albumin (g/dL)	4.7 ± 0.4	4.7 ± 0.3	4.9 ± 0.3	5.0 ± 0.3
A/G ratio	2.86 ± 0.24	2.72 ± 0.33	2.96 ± 0.42	4.08 ± 1.08##
Inorganic phosphate (mg/dL)	5.5 ± 0.9	6.1 ± 0.8	5.9 ± 0.8	6.1 ± 0.5
Ca (mg/dL)	10.3 ± 0.3	10.4 ± 0.3	10.6 ± 0.2	10.6 ± 0.3
Mg (mg/dL)	2.2 ± 0.1	2.2 ± 0.1	2.3 ± 0.1	2.4 ± 0.2
Na (mmol/L)	140.9 ± 1.0	140.4 ± 0.9	140.3 ± 1.1	139.0 ± 2.3
K (mmol/L)	4.12 ± 0.33	4.31 ± 0.18	4.19 ± 0.37	4.05 ± 0.35
Cl (mmol/L)	103.8 ± 1.2	104.0 ± 0.9	103.2 ± 0.6	97.5 ± 3.7##

Data are presented as mean ± SD.

AST: aspartate aminotransferase; ALT: alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase.

Number in parentheses indicates the number of animals examined since the values under the measuring limit were omitted.

Number in square brackets indicates the number of animals examined since the poor state samples were omitted.

*, **: Significantly different from the control group at $p < 0.05$, 0.01 (Dunnett test), respectively.

#, ##: Significantly different from the control group at $p < 0.05$, 0.01 (Steel test), respectively.

Table 7. Absolute organ weight data of rats administered Monascus Color Y-001 for 90-day.

	Dose (mg/kg/day)			
	Control	100	300	1000
[male]				
Body weight (g)	513.3 ± 49.5	541.3 ± 51.7	526.4 ± 62.7	447.1 ± 23.0*
Brain (g)	2.184 ± 0.107	2.204 ± 0.118	2.174 ± 0.042	2.146 ± 0.089
Heart (g)	1.584 ± 0.177	1.620 ± 0.147	1.577 ± 0.198	1.379 ± 0.120*
Lungs (g)	1.490 ± 0.113	1.502 ± 0.122	1.441 ± 0.107	1.395 ± 0.091
Liver (g)	11.816 ± 1.563	12.860 ± 2.272	11.923 ± 2.203	9.617 ± 0.476##
Kidneys (g)	3.250 ± 0.253	3.289 ± 0.335	3.219 ± 0.297	2.949 ± 0.260
Spleen (g)	0.793 ± 0.150	0.787 ± 0.088	0.778 ± 0.149	0.598 ± 0.056##
Thymus (g)	0.277 ± 0.082	0.268 ± 0.086	0.288 ± 0.112	0.243 ± 0.036
Pituitary gland (mg)	14.9 ± 3.4	12.8 ± 1.7	13.0 ± 1.8	14.3 ± 1.2
Thyroids (mg)	27.1 ± 6.1	29.6 ± 5.2	26.9 ± 4.7	26.5 ± 2.7
Adrenal glands (mg)	60.7 ± 10.0	68.1 ± 11.3	65.8 ± 8.6	65.1 ± 12.4
Salivary glands (g)	0.772 ± 0.091	0.792 ± 0.067	0.730 ± 0.069	0.740 ± 0.077
Testes (g)	3.465 ± 0.413	3.331 ± 0.262	3.346 ± 0.278	3.512 ± 0.585
Prostate (g)	2.245 ± 0.246	2.068 ± 0.229	2.058 ± 0.367	1.843 ± 0.316*
Epididymides (g)	1.408 ± 0.189	1.416 ± 0.142	1.400 ± 0.072	1.352 ± 0.109
Seminal vesicles (g)	1.506 ± 0.177	1.395 ± 0.153	1.479 ± 0.073	1.307 ± 0.204
[female]				
Body weight (g)	304.3 ± 20.2	295.7 ± 26.3	295.0 ± 23.8	273.8 ± 18.1*
Brain (g)	1.989 ± 0.093	1.979 ± 0.094	1.990 ± 0.088	1.959 ± 0.069
Heart (g)	0.948 ± 0.040	0.962 ± 0.089	0.978 ± 0.070	0.866 ± 0.077*
Lungs (g)	1.079 ± 0.080	1.099 ± 0.059	1.085 ± 0.071	1.020 ± 0.078
Liver (g)	6.988 ± 0.452	6.940 ± 0.901	6.886 ± 0.812	7.723 ± 0.708
Kidneys (g)	1.944 ± 0.152	1.877 ± 0.135	1.926 ± 0.137	1.841 ± 0.143
Spleen (g)	0.483 ± 0.059	0.486 ± 0.052	0.460 ± 0.052	0.414 ± 0.080*
Thymus (g)	0.270 ± 0.048	0.255 ± 0.034	0.243 ± 0.054	0.165 ± 0.066**
Pituitary gland (mg)	20.3 ± 3.8	19.6 ± 4.7	20.5 ± 3.5	17.2 ± 2.3
Thyroids (mg)	28.4 ± 5.1	27.5 ± 4.8	26.9 ± 6.6	29.2 ± 4.4
Adrenal glands (mg)	70.2 ± 11.2	71.2 ± 13.7	67.9 ± 7.1	67.8 ± 7.0
Salivary glands (g)	0.443 ± 0.040	0.433 ± 0.040	0.434 ± 0.052	0.445 ± 0.043
Ovaries (mg)	125.7 ± 13.0	118.2 ± 21.5	116.7 ± 14.6	122.2 ± 13.8
Uterus (g)	0.682 ± 0.208	0.654 ± 0.191	0.636 ± 0.185	0.638 ± 0.186

Data are presented as mean ± SD.
*, **: Significantly different from the control group at $p < 0.05$, 0.01 (Dunnett test), respectively.
##: Significantly different from the control group at $p < 0.01$ (Steel test).

et al., 2005). Although there was no evidence to suggest hemorrhage and/or hepatic dysfunction in the study, thorough attention should be paid to this finding in the subsequent longer-term study.

Treatment-related histopathological changes were observed in thymus, liver and kidney only in the high-dose females. Decreased cellularity of lymphocytes was observed in the thymic cortex with decreased thymus weight. The finding is commonly encountered in animals with nonspecific stress due to poor physiological and/or nutritional condition (Pearse, 2006), and the change is considered to be not a direct test-article effect. Centrilobular hepatocellular hypertrophy was accompanied with increased liver weight. The hepatic finding, therefore, was not considered a toxicological effect, and was most likely an adaptive change associated with hepatic drug metabolizing enzyme induction (Hall *et al*., 2012; Yoshida *et al*., 2015). Vacuolation was observed in proximal tubules of the kidneys. Some parameter changes in urinalysis and clinical biochemistry may be correlated with histopathological changes in the kidneys. Monascus colorants may contain some by-products, such as the mycotoxin citrin-

Table 8. Organ to body weight ratio data of rats administered Monascus Color Y-001 for 90-day.

	Dose (mg/kg/day)			
	Control	100	300	1000
[male]				
Brain	0.429 ± 0.046	0.410 ± 0.039	0.418 ± 0.046	0.481 ± 0.019*
Heart	0.310 ± 0.039	0.300 ± 0.015	0.300 ± 0.022	0.308 ± 0.021
Lungs	0.292 ± 0.022	0.278 ± 0.014	0.276 ± 0.029	0.312 ± 0.010#
Liver	2.297 ± 0.142	2.363 ± 0.235	2.255 ± 0.212	2.155 ± 0.134
Kidneys	0.636 ± 0.053	0.608 ± 0.034	0.615 ± 0.051	0.659 ± 0.036
Spleen	0.155 ± 0.028	0.146 ± 0.010	0.148 ± 0.021	0.134 ± 0.012
Thymus	0.054 ± 0.015	0.049 ± 0.015	0.054 ± 0.018	0.055 ± 0.008
Pituitary gland ($\times 10^{-3}$)	2.95 ± 0.84	2.37 ± 0.31	2.49 ± 0.35	3.19 ± 0.16
Thyroids ($\times 10^{-3}$)	5.25 ± 0.88	5.47 ± 0.93	5.14 ± 0.91	5.92 ± 0.56
Adrenal glands ($\times 10^{-3}$)	11.90 ± 2.04	12.61 ± 1.87	12.64 ± 2.02	14.55 ± 2.64*
Salivary glands	0.151 ± 0.020	0.147 ± 0.013	0.140 ± 0.020	0.165 ± 0.011
Testes	0.681 ± 0.103	0.620 ± 0.068	0.641 ± 0.063	0.789 ± 0.146
Prostate	0.441 ± 0.063	0.384 ± 0.050	0.400 ± 0.103	0.413 ± 0.073
Epididymides	0.277 ± 0.042	0.264 ± 0.038	0.269 ± 0.028	0.303 ± 0.026
Seminal vesicles	0.296 ± 0.042	0.260 ± 0.037	0.285 ± 0.034	0.292 ± 0.044
[female]				
Brain	0.657 ± 0.060	0.672 ± 0.046	0.679 ± 0.070	0.718 ± 0.056
Heart	0.312 ± 0.016	0.325 ± 0.016	0.332 ± 0.020*	0.316 ± 0.020
Lungs	0.355 ± 0.028	0.374 ± 0.026	0.369 ± 0.025	0.373 ± 0.018
Liver	2.302 ± 0.152	2.343 ± 0.150	2.329 ± 0.116	2.824 ± 0.226**
Kidneys	0.642 ± 0.076	0.637 ± 0.042	0.654 ± 0.038	0.673 ± 0.038
Spleen	0.159 ± 0.017	0.165 ± 0.020	0.157 ± 0.017	0.151 ± 0.024
Thymus	0.089 ± 0.019	0.087 ± 0.011	0.083 ± 0.019	0.060 ± 0.023**
Pituitary gland ($\times 10^{-3}$)	6.64 ± 1.01	6.62 ± 1.45	6.96 ± 1.23	6.28 ± 0.68
Thyroids ($\times 10^{-3}$)	9.34 ± 1.60	9.35 ± 1.73	9.12 ± 2.26	10.73 ± 1.86
Adrenal glands ($\times 10^{-3}$)	23.05 ± 3.19	24.06 ± 3.96	23.17 ± 3.26	24.81 ± 2.43
Salivary glands	0.146 ± 0.015	0.147 ± 0.015	0.148 ± 0.017	0.163 ± 0.013*
Ovaries ($\times 10^{-3}$)	41.31 ± 3.20	40.13 ± 7.43	39.92 ± 6.84	44.67 ± 4.47
Uterus	0.226 ± 0.074	0.223 ± 0.070	0.218 ± 0.069	0.232 ± 0.058

Data are presented as mean ± SD.
Above values were calculated as organ weight (g) / 100 g body weight.
*, **: Significantly different from the control group at $p < 0.05$, 0.01 (Dunnett test), respectively.
#: Significantly different from the control group at $p < 0.05$ (Steel test).

in. Citrinin has been reported to induce proximal tubular injury in the several species including rats (de Oliveira Filho *et al*., 2017; Flajs and Peraica, 2009), although the mechanism of vacuolation is unclear. However, Monascus Color Y-001 did not contain detectable citrinin, and the renal changes seen in this study were therefore not attributable to this impurity. Importantly, we note that Monascus Color Y-001 treatment has never caused any degeneration/necrosis in liver and kidneys in rats even at the 1000 mg/kg/day dose for 90 days.

Monascus Color Y-001 was administered orally by gavage to male and female SD rats at doses of 100, 300, and 1000 mg/kg/day for 90 days. Although well tolerated, various toxicologic findings were observed in both sexes at 1000 mg/kg/day. In conclusion, the no-observed-adverse-effect level (NOAEL) was judged to be 300 mg/kg/day both in male and female rats.

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Table 9. Histopathological findings in rats administered Monascus Color Y-001 for 90-day. No treatment-related changes were noted other than bellow organs.

Dose (mg/kg/day)	Male				Female			
	Control	100	300	1000	Control	100	300	1000
No. of animals	10	10	10	10	10	10	10	10
Liver								
Normal	6	6	10	10	9	9	9	0
Hypertrophy, hepatocellular, centrilobular (2)	0	0	0	0	0	0	0	10**
Infiltration, mononuclear (1)	4	3	0*	0*	1	0	0	3
Inflammatory cell infiltrate, perivascular (1)	0	1	0	0	0	0	0	0
Necrosis, focal (2)	1	0	0	0	0	0	0	0
Tension lipidosis (1)	0	0	0	0	1	1	0	0
(2)	0	0	0	0	0	0	1	0
Kidney								
Normal	8	10	10	9	9	9	9	0
Basophilia, tubule (1)	2	0	0	1	0	0	0	0
Cyst, cortex (1)	0	0	0	0	1	0	0	0
Cyst, medulla (1)	0	0	0	0	0	1	1	0
(2)	0	0	0	0	1	0	0	0
Infiltrate, inflammatory cell, interstitium (1)	0	0	0	0	0	1	0	0
Vacuolation, proximal tubules (1)	0	0	0	0	0	0	0	10**
Thymus								
Normal	9	-	-	9	10	-	-	6
Cellularity, decreased, lymphocyte (1)	0	-	-	0	0	-	-	2*
(2)	0	-	-	0	0	-	-	2*
Hemorrhage (1)	1	-	-	1	0	-	-	0
Stomach								
Normal	10	10	10	10	10	8	10	7
Erosion, glandular (2)	0	0	0	0	0	2	0	1
(3)	0	0	0	0	0	0	0	1
Ulcer, glandular (3)	0	0	0	0	0	0	0	1

Values are number of animals with findings.
Numbers in parenthesis indicate the grades of lesion: (1) Minimal, (2) Slight, (3) Moderate, (4) Marked, (5) Severe.
-: Not examined.
*, **: Significantly different from the control group at $p < 0.05$, 0.01 (Wilcoxon test), respectively.

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

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