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Original Article

Subchronic oral toxicity study of Monascus Color in beagle dogs

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ABSTRACT — Monascus Color Y-001, a natural food pigment produced from *Monascus purpureus* Y-001 fermentation, was administered orally by gavage to 4 Beagle dogs/sex/group for 90 days at doses of 0 (vehicle: 0.1% Tween 80, 10 mL/kg bw), 1, 5 and 25 mg/kg bw/day. In the clinical observations, vomiting/vomitus (dark red watery substance or undigested feed) was observed for males and females in the 25, but not 5 or 1 mg/kg bw/day group at a relatively high frequency. In addition, loose stools were observed for males in this group on a single occasion. These signs were considered to be effects of the test article on the digestive tract. No treatment-related effects were noted in the detailed observations for symptoms, body weights, food consumptions, ophthalmology, hematology, clinical chemistry, urinalysis, gross pathology, organ weights or histopathology. Thus, the no-observed-adverse-effect level (NOAEL) was judged to be 5 mg/kg bw/day in both sexes of the dogs.

Key words: Monascus Color Y-001, Subchronic toxicity, Dog

INTRODUCTION

Monascus Color Y-001, one of the Monascus colors produced from *Monascus purpureus* Y-001 fermentation, is a natural food pigment that is widely used in food industries, especially in Japan (Feng *et al*., 2012). Monascus colors formed by Monascus spp. are not only present in free form but also bound as complexes to proteins, peptides, and amino acids (Woo *et al*., 2014). The main coloring components are the two red pigments rubropunctamine and monascorubramine, the orange-red pigments rubropunctatin and monascorubrin, and the yellow pigments monascin and ankaflavin (SKLM, 2013). Kumari *et al*. (2009) published the results of acute and subchronic toxicity studies of Monascus purpureus MTCC 410-fermented rice (red mould rice; RMR) (no descriptions concerning monascus color pigments) in rats: there was no acute toxicity at 5.0 g/kg bw and no subchronic toxicity at dietary concentrations up to 12.0%. In addition, a carcinogenicity study of monascus color (no details of components) in F344 rats has been published: no tumor development was observed in rats at dietary concentrations of 1.25% and 2.5% for 2 years (Hiasa *et al*., 1997).

Monascus Color Y-001 contains two pigments bound to leucine, N-leucyl monascorubrin and N-leucyl rubropunctatin (Woo *et al*., 2014). Byproducts unsuitable for food color such as the antihypercholesterolemic agent monacolin K (EFSA, 2018) and the nephrotoxic mycotoxin citrinin (EFSA, 2012) are below detectable levels. The toxicological effects of Monascus Color Y-001 have been investigated as part of the safety assessment of this pigment as a food additive. A genotoxicity study concluded that Monascus Color Y-001 does not possess

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any genotoxic risk in humans (Sato *et al*., 2021). Results of a 90-day oral repeated-dose toxicity study of Monascus Color Y-001 in rats have been reported, and the no-observed-adverse-effect level (NOAEL) was determined to be 300 mg/kg bw/day in both male and female rats (Doi *et al.*, 2021).

The objectives of the present investigation were to evaluate the subchronic toxicological potential of Monascus Color Y-001 given orally (by gavage) for 90 days to male and female Beagle dogs.

MATERIALS AND METHODS

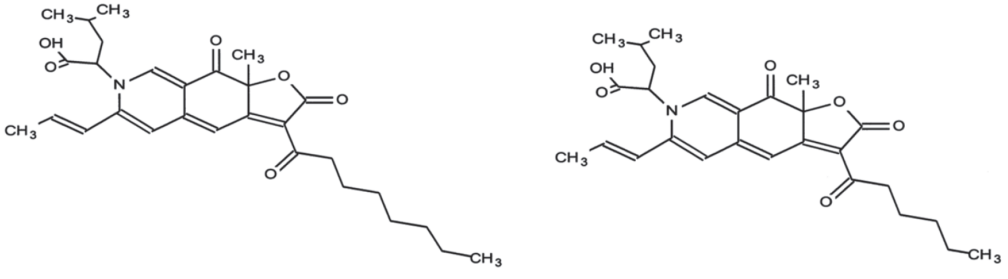
The study was conducted at Ina Research, Inc. (Nagano, Japan), in compliance with Good Laboratory Practice (GLP) Standards for National Safety Studies of Drugs (Ministry of Health and Welfare Ordinance No. 21, Mar, 26, 1997, Japan), and GLP for Nonclinical Laboratory Studies (Food and Drug Administration, 21 CFR, Part 58, Dec, 22, 1978, USA). The present study was performed in accordance with Partial Revision of the Guidelines for Repeated Dose Toxicity Studies (Notification No. 655, Apr, 5, 1999, Ministry of health and Welfare, Japan, and "Redbook 2000: IV.C.4.b. Subchronic Toxicity Studies with Non-Rodents" U.S. Food and Drug Administration, November, 2003). The present study was conducted in accordance with the "Act on Welfare and Management of Animals" in Japan, and the "Guidance for Animal Care and Use" of Ina Research Inc. and in accordance with the protocol reviewed by the Institutional Animal Care and Use Committee (IACUC) of Ina Research Inc., which is fully accredited by AAALAC International (Accredited Unit No. 001107).

Test material and vehicle

Monascus Color Y-001, provided by YAEGAKI Bio-industry, Inc. (Himeji, Japan), is dark red powder having a unique odor. The main components of the Monascus Color Y-001 are N-leucyl monascorubrin and N-leucyl rubropunctatin (Fig. 1). The pigment components of the Monascus Color Y-001 used in the present study (Lot No. 20211026) were 51.9% N-leucyl monascorubrin and 33.1% N-leucyl rubropunctatin. The content of fermentation byproducts monacolin K and citrinin were below their detection limits of 10 μg/g and 0.2 μg/g, respectively. The other components accounted for less than 10% of the total weight of the Monascus Color Y-001. The pigmentation components of the Monascus Color Y-001 used in the preliminary study (Lot No. 20210624) were 56.7% N-leucyl manascorubrin and 37.9% N-leucyl rubropunctatin. 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 the vehicle. Monascus Color Y-001 was suspended in 0.1% Tween 80 for each dosage level.

Animals and husbandry

Four-month-old male and female Beagle dogs were purchased from Kitayama Labes Co. Ltd. (Kyoto, Japan) and acclimated for at least 13 days before allocation into groups. Dogs were individually housed in built-in concrete/stainless steel dog cages (80W × 92D × 95H) in an animal facility with a temperature of 20 – 26ºC, humidity of 40.0 – 70.0%, ventilation frequency of 23 – 27 times clean, fresh air changes/hour, and a 12-hr light/dark cycle (7:00 to 19:00). DS-A pelleted feed (Oriental Yeast Co., Ltd., Tokyo, Japan) and Ina City tap water were available *ad libitum*. Group allocation of dogs was conducted using



N-leucyl monascorubrin N-leucyl rubropunctatin

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

a computer system (Provantis) in an attempt to avoid familial biases among the groups. Animals were stratified based on the body weights measured on the day of group allocation. Animals were then selected from each stratum and randomly distributed into groups.

Preliminary 4-week range finding study

Eight male and 8 female Beagle dogs were allocated to 4 groups using a computer system (Provantis) in an attempt to avoid familial biases among the groups. Animals were stratified based on the body weights measured on the day of group allocation. The animals were administered dosing solutions (dose volume: 5 mL/kg bw/day) at dose levels of 0 (vehicle), 150, 300 and 600 mg/kg bw/day for males on day 1 and 2. However, one male in the 300 mg/kg bw/day group died on day 1 and one male in the 600 mg/kg bw/day group died on day 2. Therefore, dose levels were reduced to 50, 75 and 100 mg/kg bw/day from day 3 for males: 2 males in the control group were reallocated to the 50 mg/kg bw/day group, 2 males in the 150 mg/kg bw/day group were reallocated to the 75 mg/kg bw/day group, and the one surviving male in the 300 mg/kg bw/day group and the one surviving male in the 600 mg/kg bw/day group were reallocated to the 100 mg/kg bw/day group. Females were treated with dosing solutions (dose volume 5 mL/kg bw/day) at dose levels of 0 (vehicle), 50, 75, and 100 mg/kg bw/day from day 1.

In the 2 male animals that died, hemorrhage and degeneration/necrosis of the mucosa of the gastrointestinal tract were observed histopathologically. In these animals, hemorrhage was also observed sporadically in other organs, indicating a bleeding tendency.

After changing the dose levels, vomitus (undigested feed or dark red watery substance) was observed almost daily for both males and females in the 50, 75, and 100 mg/kg bw/day groups and no appreciable differences were noted among the groups. In addition, test article-colored (dark red) watery stools were observed for 1 male in the 100 mg/kg bw/day group. Transiently decreased food consumption was noted in females in the 100 mg/kg bw/day group on Day 27; however, this change did not affect the body weights. No treatment-related effects were noted in the body weights, ophthalmology, hematology, clinical chemistry, urinalysis, gross pathology, organ weights or histopathology.

Based on the above results and reasoning that the clinical signs observed above were treatment-related effects on the digestive tract, a dose level of less than 50 mg/kg bw/day was concluded to be appropriate for a 13-week repeated dose toxicity study.

Subchronic oral toxicity study (gavage study)

Based on the results of a 4-week range finding study discussed above, the highest dose level was set at 25 mg/kg bw/day for the current study, and 5 and 1 mg/kg bw/day were selected as the mid and low dose levels. The study design is shown in Table 1. The animals were treated orally by gavage once daily for 90 days, and the dosing volume (1 mL/kg body weight) was adjusted throughout the study to the most recent body weight measurement.

Observations and Examinations

All animals were checked three times daily (pre-dosing, and 1 and 6 hr post–dosing) for clinical observations. During the clinical observations the animals were carefully observed for changes in the skin, fur, eyes, and mucosa; the presence of discharge or excrement; gait, posture, reaction to handling, clonic and tonic convulsions, stereotype (excessive grooming, circling, etc.), and abnormal behavior (self-harming behavior, backward walking etc.); and other autonomic nervous activity (salivation, piloerection, and respiration). Body weights were measured during the pretest period (2 times) and weekly during the dosing period. Food consumption (provided and residual amounts of feed were measured) was calculated daily from the pretest period to 1 day prior to necropsy.

Ophthalmological examinations were performed during the pretest period and on week 13: all animals in all groups were examined during the pretest period; animals in the control and high dose groups were examined on week 13. Ophthalmological examination con-

Table 1. Dosage group design in dogs administered Monascus Color Y-001 for 90 days.

Test article	Dose (mg/kg bw/day)	Dose volume (mL/kg bw)	Concentration (mg/mL)	Number of dogs	
				Male	Female
Control (vehicle)a	0	1	0	4	4
Monascus Color Y-001	1	1	1	4	4
	5	1	5	4	4
	25	1	25	4	4

a: 0.1% Tween 80 in distilled water.

sisted of examination of the anterior portions of the eyes, optic media, and ocular fundi using a slit lamp and a binocular indirect ophthalmoscope: the optic media and ocular fundi were examined after administration of a mydriatic (Mydrin-P, Santen Pharmaceutical Co. Ltd., Osaka, Japan).

During the pretest period and at weeks 2, 7, and 13, blood samples were collected via the cephalic vein from all animals. For hematology, hematological estimations were carried out using an automated hematology system, XN-2000 (Sysmex Co., Kobe, Japan), for the red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentrtion (MCHC), reticulocyte ratio (RET%), reticulocyte count (RET#), platelet count (PLT), white blood cell count (WBC), differential white blood cell ratio, and count (Diff WBC% and #), neutrophils (NEUT), lymphocytes (LYMPH), monocytes (MONO), eosinophils (EO) and basophils (BASO)). Coagulation times were measured using an automated blood coagulation analyzer, CA-510 (Sysmex Co., Kobe, Japan), for the proyhrombin time (PT) and the activated partial thromboplastin time (APTT).

For clinical biochemistry, serum samples were obtained by centrifuging clotted blood samples, and evaluation was performed using a clinical analyzer model 7180 (Hitachi High-technologies Co., Ltd, Tokyo, Japan). Parameters measured were aspartate aminotranferase (AST) alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LD), creatine kinase (CK), glucose (GLU), total bilirubin (BIL), urea nitrogen (UN), creatinine (CRE), total cholesterol (CHO), total bile acid (T-BA), triglyceride (TG), phospholipid (PL), inorganic phosphorus (IP), calcium (CA), sodium (NA), potassium (K), chloride (CL), total protein (TP), albumin (ALB), globulin (GLOB), and the albumin-globulin ratio (A/G).

Urinalysis performed pretest period, and weeks 5, 9 and 13 for all animals. Urine collection was started immediately after dosing. Urine collection during the pretest period was started at the time corresponding to the sampling time during week 13. Fresh urine samples (4 mL) were collected by pipetting from urine at 4-hr of urine collection. Using the frsh urine samples, sediments were examined by microscoy after Sternheimer staining, and parameters on the reagent strips (Multistix SG-L, Siemens Healthcare Diagnostics K.K. Tokyo, Japan) were analyzed using Urine Chemistry Analyzer Clinic Advantus (Siemens Healthcare Diagnostics K.K., Tokyo, Japan).Total urine volume was measured 24-hr urine outputs. Using the 24-hr cumulative urine samples, following tests were performed: urine color by gross observation, specific gravity by Urine S.G. Refractometer URC-JE (Atago Co. Ltd., Tokyo, Japan), and electrolytes were measured by Hitachi 7180.

Postmortem examinations

All animals were anesthetized by intravenous injection of thiopental sodium (RAVONAL, Nipro ES Pharma Co. Ltd., Osaka, Japan), euthanized by exsanguination from the axillary and femoral arteries and veins, and subjected to necropsy. Heart, aorta (thoracic), sternum (bone marrow), femurs (bone marrow), thymus, spleen, lymph node (submandibular, mesenteric), trachea, bronchi, lungs, tongue, submandibular glands, parotid glands, esophagus, stomach, duodenum, jejunum, ileum, Peyer's patches, cecum, colon, rectum, liver, gallbladder, pancreas, kidneys, urinary bladder, pituitary, thyroids, adrenals, testes, epididymides, prostate, ovaries, oviducts, uterus, vagina, brain, spinal cord (cervical, thoracic and lumbar regions), sciatic nerves, eyes, optic nerves, lacrimal glands, nasal cavity (nasoturbinate), skeletal muscle (thigh), skin (abdominal), mammary glands, and organs/tissues with gross lesions were excised, fixed in 10% buffered formalin solution and processed for histopathological examination. Testes were initially fixed in formalin-sucrose-acetic acid (FSA) solution, and post-fixed in in 10% neutral buffered formalin. Eyes with optic nerves were initially fixed in 1% formaldehyde-2.5% glutaraldehyde in phosphate buffer, and post-fixed in 10% neutral buffered formalin. Heart, thymus, spleen, lung with bronchi, submandibular glands, liver with gallbladder, pancreas, kidneys, pituitary, thyroids with parathyroids, adrenals, testes, epididymides, prostate, ovaries, uterus, and brain were weighed using an electronic balance (UX620H, Shimadzu Co. Ltd., Kyoto, Japan), and organ-to body weight ratios were determined. A full Histopathological examination was performed on hematoxylin and eosin-stained tissue sections of the above organs and tissues from the control and highest dose group dogs.

Statistical analysis

The numerical data were assessed using Dunnett's test. Significant differences between the control and test material groups were analyzed and evaluated at $p < 0.05$ or $p < 0.01$.

RESULTS

Mortality

There were no deaths at any of the doses used in the

Table 2. Body weights and food consumption data on the first day (Day 1) and the final day (Day 90) of dogs administered Monascus Color Y-001 for 90 days.

	Dose (mg/kg bw/day)			
	Control	1	5	25
[male]				
Body weights (kg)				
Day 1	7.29 ± 0.63	7.38 ± 0.59	7.37 ± 0.33	7.38 ± 0.55
Day 90	9.62 ± 0.82	9.93 ± 0.86	9.42 ± 0.77	9.62 ± 1.55
Food consumption (g/animal/day)				
Day 1	300 ± 0	300 ± 0	300 ± 0	300 ± 0
Day 90	300 ± 0	300 ± 0	300 ± 0	300 ± 0
[female]				
Body weights (kg)				
Day 1	6.83 ± 0.23	7.05 ± 0.38	7.01 ± 0.41	6.90 ± 0.27
Day 90	8.70 ± 0.41	8.93 ± 0.77	8.70 ± 0.47	8.85 ± 0.77
Food consumption (g/animal/day)				
Day 1	300 ± 0	300 ± 0	300 ± 0	300 ± 0
Day 90	300 ± 0	300 ± 0	300 ± 0	300 ± 0

Data are presented as mean ± SD.

study during the treatment period.

Clinical Observations

A relatively high frequency of vomiting/vomitus (dark red watery substance or undigested feed) was observed for 3 males and 2 females in the 25 mg/kg bw/day group. In addition, loose stools were observed for 2 males in this group on a single occasion. These signs were suggestive of effects of Monascus Color Y-001 on the digestive tract. In the 5 mg/kg bw/day group, vomitus (dark red watery substance or undigested feed) was observed for 3 males and 1 female. This was not considered to be a toxic finding since the frequency observed during the dosing period was only once for each animal, which is comparable to the frequency of spontaneous occurrence. No treatment-related effects were noted in the 1 mg/kg bw/day group. In all treated groups, Monascus Color Y-001-colored stools were observed dose-dependently for both males and females. However, this was not considered to be toxicologically significant since the stools were the color of the excreted Monascus Color Y-001. Detailed clinical observations of the dogs found no other treatment-related effects in any group. No treatment-related effects on body weight or food consumption were noted in any group (Table 2).

In ophthalmology, no treatment-related effects were observed in the 25 mg/kg bw/day group. White dots in the lens (anterior cortex) and depigmented areas on the retina were observed in the 25 mg/kg bw/day group at week 13. However, these findings were not considered to be treatment-related since they occur spontaneously in dogs and were present on examination prior to treatment initiation.

In hematology, no treatment-related effects were noted in any group. Statistically significant lower values in MCV and higher values in RBC, HGB, and HCT were noted in females in the 1 and 5 mg/kg bw/day groups at weeks 2, 7, and 13 (Table 3) compared to the control values. However, these changes were considered to be incidental since they were not dose related, and they were also observed in the pretest period.

In clinical chemistry, no treatment-related effects were noted in any group (Tables 4 and 5). Statistically significant lower values in ALB were noted in males in the 5 and 25 mg/kg bw/day group at weeks 2 and 13 (Table 4) compared to the control values. However, ALB values in these groups tended to be lower in the pretest period, and no significant changes were noted in any related parameters such as TP. Therefore, these significant differences were considered to be incidental changes. Significant changes in ALP, LD, CK, CHO, T-BA, TG, CA, and K were also observed in the current study, however, these changes were not dose-related and were transient.

In urinalysis, no treatment-related effects were noted in any group. A protein positive response (+), was noted in a male in the 25 mg bw/kg/day group at week 5. However, this positive response was considered not to be treatment related since this change was observed in only one animal and was transient.

Postmortem examinations

In gross pathology, no treatment-related gross lesions were observed in any animal. A cyst in the cortex of the

Table 3. Hematology data of dogs administered Monascus Color Y-001 for 90 days.

		Dose (mg/kg bw/day)			
	Weeks at	Control	1	5	25
[male][a]					
[female][b]					
RBC ($\times 10^6$μL)	-3	5.87 ± 0.06	6.62 ± 0.40**	6.90 ± 0.14**	6.09 ± 0.13
	2	6.41 ± 0.35	6.78 ± 0.18	7.32 ± 0.16**	6.39 ± 0.16
	7	6.82 ± 0.39	7.11 ± 0.20	7.58 ± 0.18**	6.97 ± 0.36
	13	6.74 ± 0.36	7.58 ± 0.41**	7.79 ± 0.45**	7.10 ± 0.35
HGB (g/dL)	-3	13.3 ± 0.4	14.3 ± 0.6	14.8 ± 0.3**	13.4 ± 0.3
	2	14.6 ± 0.5	14.9 ± 0.4	16.0 ± 0.3**	14.3 ± 0.7
	7	15.4 ± 0.7	15.6 ± 0.4	16.6 ± 0.4	15.7 ± 1.5
	13	15.1 ± 0.6	16.5 ± 0.8	16.8 ± 0.8	15.8 ± 1.6
HCT (%)	-3	39.7 ± 0.8	42.9 ± 1.5	43.9 ± 0.9**	39.9 ± 0.6
	2	43.0 ± 1.9	43.9 ± 1.2	46.5 ± 0.7**	41.7 ± 1.3
	7	45.4 ± 2.6	45.8 ± 1.0	48.0 ± 0.8	45.6 ± 3.4
	13	44.5 ± 2.0	48.0 ± 2.4	48.8 ± 2.1	46.1 ± 3.4
MCV (fL)	-3	67.7 ± 0.8	64.9 ± 1.9*	63.6 ± 0.6**	65.5 ± 1.1
	2	67.2 ± 0.9	64.7 ± 2.0	63.6 ± 0.4*	65.3 ± 1.7
	7	66.6 ± 0.9	64.4 ± 1.7	63.3 ± 0.9*	65.4 ± 1.9
	13	66.0 ± 0.9	63.3 ± 1.6*	62.7 ± 0.8*	64.8 ± 1.9
MCH (pg)	-3	22.7 ± 0.5	21.7 ± 1.0	21.5 ± 0.3*	22.1 ± 0.5
	2	22.8 ± 0.8	22.0 ± 0.8	21.8 ± 0.4	22.4 ± 1.2
	7	22.6 ± 0.5	22.0 ± 0.6	21.9 ± 0.3	22.5 ± 1.3
	13	22.5 ± 0.4	21.9 ± 0.6	21.6 ± 0.3	22.2 ± 1.3
RET #. (10^9/L)	-3	98.4 ± 26.1	69.8 ± 23.6	71.5 ± 15.5	55.5 ± 13.6*
	2	61.8 ± 6.7	72.1 ± 27.1	74.0 ± 16.0	56.7 ± 20.9
	7	49.5 ± 9.8	39.8 ± 8.6	43.5 ± 8.6	38.7 ± 17.1
	13	46.9 ± 12.4	41.3 ± 15.3	44.5 ± 14.8	32.3 ± 19.3
Differential white blood cells					
NEUT # (10^3/μL)	-3	5.82 ± 0.58	4.41 ± 0.44*	5.96 ± 0.85	4.37 ± 0.66*
	2	5.00 ± 1.17	4.30 ± 0.83	5.34 ± 0.12	4.01 ± 0.62
	7	4.74 ± 0.80	4.33 ± 1.38	5.77 ± 1.27	4.23 ± 1.03
	13	5.26 ± 0.74	4.36 ± 0.25	5.10 ± 0.27	5.36 ± 2.60
EOS # (10^3/μL)	-3	0.12 ± 0.04	0.20 ± 0.06	0.26 ± 0.08**	0.12 ± 0.01
	2	0.15 ± 0.11	0.26 ± 0.10	0.22 ± 0.09	0.17 ± 0.07
	7	0.14 ± 0.12	0.24 ± 0.05	0.40 ± 0.31	0.17 ± 0.05
	13	0.24 ± 0.33	0.21 ± 0.08	0.21 ± 0.06	0.14 ± 0.01

Data are presented as mean ± SD. No. of animals examined were 4/sex/group.

[a]: No statistically significant changes were found in male dogs at any parameter and interval.

[b]: In female dogs, parameters (MCHC, PLT, WBC, LYMPH #, MONO #, BASO #, PT and APTT) did not show significant changes were excluded from this tables.

Abbreviations: See section of"Observations and Examinations".

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

left kidney was observed in a male in the control group.

In organ weights, no treatment-related changes were noted in any group. Statistically significant lower values in organ to body weight ratio of the adrenals were noted in males in the 25 mg/kg bw/day group (Table 6). Organ weight changes in the adrenals were considered to be incidental and not treatment related since the individual values were within the range of historical data of this laboratory, and no alterations were found histopathologically.

No treatment-related histopathological lesions were observed in any animal. The cyst in the kidney observed

Table 4. Clinical biochemistry data of male dogs administered Monascus Color Y-001 for 90 days.

		Dose (mg/kg bw/day)			
	Week at	Control	1	5	25
[male][a]					
ALP (U/L)	-3	682 ± 48	523 ± 56*	532 ± 111*	646 ± 71
	2	569 ± 48	422 ± 46*	456 ± 111	500 ± 76
	7	452 ± 60	355 ± 34	374 ± 96	412 ± 47
	13	385 ± 37	300 ± 33	284 ± 85	323 ± 64
LD (U/L)	-3	114 ± 58	82 ± 25	94 ± 43	83 ± 28
	2	69 ± 17	54 ± 16	79 ± 5	57 ± 14
	7	94 ± 32	50 ± 5*	72 ± 9	61 ± 15
	13	65 ± 22	57 ± 7	81 ± 13	74 ± 17
CK (U/L)	-3	349 ± 83	237 ± 40	300 ± 136	274 ± 29
	2	255 ± 40	197 ± 5*	255 ± 42	202 ± 6
	7	242 ± 30	168 ± 12**	225 ± 34	187 ± 16*
	13	181 ± 40	151 ± 20	171 ± 37	154 ± 11
CHO (mg/dL)	-3	134 ± 5	128 ± 9	131 ± 9	130 ± 23
	2	150 ± 10	129 ± 6	126 ± 15	132 ± 18
	7	166 ± 13	135 ± 20	133 ± 13	145 ± 23
	13	150 ± 12	131 ± 15	124 ± 14*	141 ± 10
TG (mg/dL)	-3	28 ± 6	20 ± 3	26 ± 11	24 ± 3
	2	19 ± 2	17 ± 2	18 ± 8	18 ± 8
	7	29 ± 7	22 ± 3	26 ± 9	25 ± 6
	13	30 ± 10	16 ± 3*	22 ± 5	21 ± 1
CA (mg/dL)	-3	10.66 ± 0.16	10.59 ± 0.22	10.72 ± 0.25	10.46 ± 0.43
	2	10.85 ± 0.09	10.67 ± 0.28	10.74 ± 0.17	10.71 ± 0.30
	7	10.97 ± 0.27	10.83 ± 0.28	10.80 ± 0.42	10.84 ± 0.08
	13	11.04 ± 0.16	10.83 ± 0.25	10.62 ± 0.17*	10.74 ± 0.19
NA (mEq/L)	-3	148.0 ± 0.6	147.6 ± 1.3	148.3 ± 1.1	147.6 ± 1.3
	2	147.7 ± 0.7	147.2 ± 1.1	147.4 ± 0.8	147.7 ± 1.2
	7	147.5 ± 1.0	148.1 ± 0.5	148.6 ± 0.3	149.1 ± 0.7*
	13	149.6 ± 1.0	149.7 ± 0.6	149.7 ± 0.9	149.4 ± 1.2
ALB (g/dL)	-3	2.51 ± 0.03	2.46 ± 0.10	2.38 ± 0.10	2.37 ± 0.13
	2	2.59 ± 0.02	2.47 ± 0.04	2.45 ± 0.10*	2.41 ± 0.08**
	7	2.72 ± 0.10	2.64 ± 0.07	2.66 ± 0.14	2.66 ± 0.11
	13	2.72 ± 0.04	2.65 ± 0.11	2.54 ± 0.10*	2.52 ± 0.10*

Data are presented as mean ± SD. No. of animals examined were 4/sex/group.

[a]: In male dogs, parameters (AST, ALT, GLU, BIL, UN, CRE, T-BA, PL, IP, K, CL, TP, GLOB, and A/G) did not show significant changes were excluded from this tables.

Abbreviations: See section of "Observations and Examinations".

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

in the gross pathology was also a cyst histopathologically, and as the cyst occurred in a control animal it was not caused by treatment with Monascus Color Y-001. No alterations were observed in the adrenals of males in the 25 mg/kg bw/day group histopathologically, although, as noted above they had significantly lower organ to body weight ratios compared to the controls. Other histopathological lesions, such as mineralization of the heart, hyperplasia of alveoli/bronchioles in the lung, and mononuclear cell infiltration in various organs/tissues, in the male and female 25 mg/kg bw/day groups (Table 7) were spontaneous changes commonly found in dogs.

DISCUSSION

In the present 90-day oral gavage study of Monascus Color Y-001 in Beagle dogs, a relatively high frequency of vomiting/vomitus (dark red watery substance or undigested feed) was observed in males and females in the 25 mg/kg bw/day group. In addition, loose stools were

Table 5. Clinical biochemistry data of female dogs administered Monascus Color Y-001 for 90 days.

		Dose (mg/kg bw/day)			
	Week at	Control	1	5	25
[female][a]					
LD (U/L)	-3	109 ± 61	110 ± 54	83 ± 16	68 ± 13
	2	100 ± 26	81 ± 11	80 ± 6	62 ± 6*
	7	74 ± 16	54 ± 5	74 ± 15	62 ± 9
	13	73 ± 26	58 ± 9	110 ± 49	62 ± 12
T-BA (μmol/L)	-3	1.2 ± 0.8	1.9 ± 2.8	3.3 ± 5.6	1.3 ± 0.8
	2	1.7 ± 1.8	0.9 ± 0.7	8.0 ± 3.4*	0.8 ± 0.7
	7	3.5 ± 3.2	3.1 ± 3.7	7.7 ± 6.6	3.4 ± 4.9
	13	2.4 ± 2.9	1.4 ± 0.4	0.6 ± 0.3	8.3 ± 14.7
TG (mg/dL)	-3	13 ± 4	23 ± 5	26 ± 10*	15 ± 5
	2	17 ± 3	20 ± 5	23 ± 12	16 ± 2
	7	21 ± 5	25 ± 11	23 ± 10	19 ± 3
	13	16 ± 4	22 ± 7	19 ± 11	14 ± 6
K (mEq/L)	-3	5.21 ± 0.15	5.00 ± 0.24	5.15 ± 0.13	4.99 ± 0.14
	2	5.32 ± 0.26	4.80 ± 0.26*	5.18 ± 0.27	4.86 ± 0.22
	7	5.03 ± 0.13	4.54 ± 0.42*	4.96 ± 0.11	4.90 ± 0.16
	13	4.71 ± 0.12	4.51 ± 0.37	4.72 ± 0.18	4.60 ± 0.30

Data are presented as mean ± SD. No. of animals examined were 4/sex/group.

[a]: In female dogs, parameters (AST, ALT, ALP, CK, GLU, BIL, UN, CRE, CHO, PL, IP, CA, NA, CL, TP, ALB, GLOB, and A/G) did not show significant changes were excluded from this tables.

Abbreviations: See section of "Observations and Examinations".

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

observed for 2 males in this group on a single occasion. These signs were considered to be effects of Monascus Color Y-001 on the digestive tract (Elwood *et al*., 2010). Detailed clinical observations of the dogs found no other treatment-related effects. There were no effects of administration of Monascus Color Y-001 on body weights, food consumptions, ophthalmology, hematology, clinical chemistry, urinalysis, gross pathology, organ weights or histopathology in any group. In the 5 mg/kg bw/day group, vomiting was observed in 3 males and 1 female, but it was considered to be an incidental finding because it occurred only once during the treatment period and the timing of occurrence varied. From these results, the NOAEL was concluded to be 5 mg/kg bw/day in male and female beagle dogs.

Treatment-related adverse effects were found in rats at a dose of 1000 mg/kg bw/day, but not at 300 mg/kg bw/day (Doi *et al*., 2021). The much lower values of the NOAEL in dogs compared to rats was related to vomiting. It is a well-known fact that the clinical symptom of vomiting is species-specific, and is observed in dogs, but not in rodents (du Sert *et al*., 2012; Horn *et al*., 2013). In a preliminary range finding study, dogs receiving 50, 75 and 100 mg/kg bw/day resulted in vomiting/vomitus almost daily for both males and females in these groups, and no appreciable differences were noted among the groups. However, no treatment-related adverse effects were noted in body weights, ophthalmology, hematology, clinical chemistry, urinalysis, gross pathology, organ weights, or histopathology.

The fermentation byproduct anticholesterolemic agent monacolin K in the Monascus Color Y-001 used in the present study was below the detection limit of 10 μg/g. This was also supported by the results of the preliminary 4-week range finding study, serum cholesterol and triglyceride values were not affected in dogs given up to 100 mg/kg bw/day Monascus Color Y-001. Similarly, these parameters were not affected in rats given up to 1000 mg/kg bw/day Monascus Color Y-001 (Doi *et al*., 2021). Furthermore, serum cholesterol and triglyceride values were not reduced in either male of female rats given commercially available monascus color (Japan's Specifications and Standards for Food Additives, 6 th edition, 1992) for 2 years (Hiasa *et al*., 1997). On the other hand, significant dose-dependent reduction of serum and liver cholesterol and triglyceride levels were reported in rats fed diets containing 2% to 12% RMR, which contained 470 μg monacolin K per gram RMR (Kumari

Table 6. Organ to body weight ratio data of dogs administered Monascus Color Y-001 for 90 days.

	Dose (mg/kg bw/day)			
	Control	1	5	25
[male]				
Brain (%)	0.879 ± 0.071	0.779 ± 0.013	0.847 ± 0.153	0.837 ± 0.155
Pituitary (mg%)	0.618 ± 0.068	0.647 ± 0.145	0605 ± 0.099	0.612 ± 0.031
Thyroids (mg%)	9.63 ± 2.52	11.43 ± 1.13	9.39 ± 2.28	8.45 ± 0.0.71
Submandibular glands (%)	0.104 ± 0.020	0.108 ± 0.008	0.099 ± 0.016	0.106 ± 0.013
Thymus (%)	0.095 ± 0.022	0.125 ± 0.039	0.123 ± 0.041	0.077 ± 0.034
Lung (%)	0.833 ± 0.049	0.800 ± 0.027	0.872 ± 0.034	0.946 ± 0.112
Heart (%)	0.788 ± 0.081	0.732 ± 0.079	0.785 ± 0.076	0.732 ± 0.054
Liver (%)	2.51 ± 0.17	2.52 ± 0.28	2.42 ± 0.21	2.49 ± 0.31
Spleen (%)	0.197 ± 0.044	0.223 ± 0.047	0.209 ± 0.033	0.183 ± 0.065
Pancreas (%)	0.238 ± 0.018	0.231 ± 0.012	0.221 ± 0.007	0.221 ± 0.009
Adrenals (mg%)	10.27 ± 1.14	8.90 ± 0.38	8.86 ± 0.78	8.42 ± 0.66*
Kidneys (%)	0.439 ± 0.056	0.410 ± 0.015	0.447 ± 0.035	0.396 ± 0.042
Testes (%)	0.144 ± 0.010	0.163 ± 0.005	0.154 ± 0.011	0.145 ± 0.024
Epididymides (%)	0.026 ± 0.005	0.024 ± 0.003	0.028 ± 0.005	0.026 ± 0.002
Prostate (%)	0.032 ± 0.007	0.032 ± 0.009	0.045 ± 0.013	0.034 ± 0.014
[female]				
Brain (%)	0.874 ± 0.036	0.828 ± 0.007	0.887 ± 0.047	0.808 ± 0.076
Pituitary (mg%)	0.711 ± 0.036	0.745 ± 0.170	0.631 ± 0.062	0.694 ± 0.120
Thyroids (mg%)	9.41 ± 3.20	8.37 ± 2.15	9.46 ± 2.71	10.76 ± 3.78
Submandibular glands (%)	0.110 ± 0.010	0.101 ± 0.008	0.100 ± 0.007	0.116 ± 0.017
Thymus (%)	0.089 ± 0.009	0.103 ± 0.024	0.091 ± 0.047	0103 ± 0.032
Lung (%)	0.839 ± 0.103	0.847 ± 0.169	0.851 ± 0.057	0.833 ± 0.075
Heart (%)	0.815 ± 0.068	0.768 ± 0.020	0.772 ± 0.057	0.793 ± 0.105
Liver (%)	2.49 ± 0.21	2.27 ± 0.08	2.54 ± 0.22	2.47 ± 0.21
Spleen (%)	0.223 ± 0.020	0.204 ± 0.042	0.224 ± 0.024	0.219 ± 0.034
Pancreas (%)	0.269 ± 0.049	0.241 ± 0.033	0.252 ± 0.040	0.283 ± 0.038
Adrenals (mg%)	9.73 ± 2.32	9.92 ± 2.07	10.30 ± 0.86	9.41 ± 1.24
Kidneys (%)	0.431 ± 0.030	0.425 ± 0.051	0.420 ± 0.010	0.416 ± 0.017
Ovaries (mg%)	7.98 ± 1.20	7.37 ± 0.93	7.20 ± 1.08	7.32 ± 1.26
Uterus (%)	0.028 ± 0.007	0.031 ± 0.010	0.034 ± 0.009	0.028 ± 0.008

Data are presented as mean ± SD. No. of animals examined were 4/sex/group.
Values are calculated as organ weight (g) / 100 g body weight.
*: Significantly different from the control group at $p < 0.05$ (Dunnett test).

et al., 2009). Monacolin K intake was approximately 898 μg/kg bw/day for males and 1048 μg/kg bw/day for females in the low dose group.

The nephrotoxic mycotoxin citrinin, another fermentation byproduct, was also below the detection limit of 0.2 μg/g in the Monascus Color Y-001 used in the present study. This was also supported by the results of the preliminary 4-week range finding study and the present subchronic toxicity study in dogs: serum clinical chemistry parameters related to renal toxicity were not affected in males or females in the 50, 75, and 100 mg/kg bw/day groups. In addition, no renal alterations were observed histopathologically. Similarly, serum biochemical parameters related to renal toxicity were not found in rats given up to 1000 mg/kg bw/day Monascus color Y-001, and no treatment-related renal lesion were found histopathologically (Doi *et al*., 2021). Notably, the NOAEL of citrinin was determined to be 20 μg/kg bw/day from a subchronic 90-day oral toxicity study in rats (EFSA 2012; Lee *et al*., 2010). However, dietary feeding of RMR, which contains 14.3 μg citrinin/g, at 2% to 12% for 14 weeks did not have any effect on serum biochemical parameters related to renal toxicity (Kumari *et al*., 2009). The citrinin intake in the Kumari *et al*. study was approximately 152.7 μg/kg bw/day for males and 192.2 μg/kg bw/day for females in the

Table 7. Histopathological findings in dogs administered Monascus Color Y-001 for 90 days.

Dose (mg/kg bw/day)	Male				Female			
	Control	1	5	25	Control	1	5	25
No. of animals examined	4	-	-	4	4	-	-	4
Heart								
Normal	4	-	-	4	3	-	-	3
Mineralization, root of aorta (p)	0	-	-	0	0	-	-	1
Infiltrate, mononuclear cell, focal (1)	0	-	-	0	1	-	-	0
Thymus								
Normal	4	-	-	4	3	-	-	4
Cyst, epithelial (p)	0	-	-	0	1	-	-	0
Submandibular lymph node								
Normal	3	-	-	4	4	-	-	4
Apoptosis, increased, lymphocyte (1)	1	-	-	0	0	-	-	0
Lung								
Normal	1	-	-	2	2		-	2
Hyperplasia, bronchiolo-alveolar (1)	0	-	-	0	0	-	-	1
Metaplasia, osseous (p)	1	-	-	0	1	-	-	0
Infiltrate, mononuclear cell focal (1)	3	-	-	2	2	-	-	1
Parotid gland								
Normal	3	-	-	4	4	-	-	3
Infiltrate, mononuclear cell focal (1)	1			0	0			1
Duodenum								
Normal	3	-	-	4	4	-	-	4
Ectopic tissue, pancreas (p)	1	-	-	0	0	-	-	0
Pancreas								
Normal	3	-	-	4	4	-	-	4
Infiltrate, mononuclear cell focal (1)	1	-	-	0	0	-	-	0
Liver								
Normal	3	-	-	3	3	-	-	3
Infiltrate, mononuclear cell focal (1)	1	-	-	1	1	-	-	1
Kidney								
Normal	3	-	-	4	3	-	-	3
Cast, hyaline (1)	0	-	-	0	1	-	-	1
Cyst (p)	1	-	-	0	0	-	-	0
Pituitary								
Normal	4	-	-	4	4	-	-	3
Cyst, pars distalis (p)	0	-	-	0	0	-	-	1
Parathyroid								
Normal	3	-	-	3	3	-	-	3
Cyst (p)	1	-	-	1	1	-	-	1
Adrenal								
Normal	3	-	-	4	4	-	-	4
Vacuolation, cortical, increased, focal (1)	1	-	-	0	0	-	-	0
Lacrymal gland								
Normal	4	-	-	3	4	-	-	3
Infiltrate, mononuclear cell focal (1)	0	-	-	1	0	-	-	1

Values are number of animals with histopathological findings.
Numbers in parenthesis indicate the grades of lesion: (p) Present, (1) Minimal..
-: Not examined.
Tissues/organs with no histopathological findings were excluded from this table.

highest dose group. Importantly, in the study by Lee *et al*. the highest dose used was the 20 μg/kg bw/day. Therefore, it is possible that the NOAEL of citrinin in rats could be considerably higher than the currently accepted 20 μg/kg bw/day, as suggested by the Kumari *et al*. study. In addition, blood urea nitrogen (BUN) values were significantly, but very slight, increased in both sexes of rats given commercially available monascus color at the 2.5% dose group (citrinin: not more than 0.2 μg/g, Japan's Specifications and Standards for Food Additives, 6 th edition, 1992) for 2 years, but no renal tumor development was observed. Taken together, these findings suggest that in the present study using Monascus Color Y-001 with citrinin below the detection limit, there was no citrinin-mediated nephrotoxicity.

In conclusion, the present results indicate that Monascus Color Y-001 at dietary levels of up to 5 mg/kg bw/day for 90 days caused no adverse effects on any of the parameters examined in either male or female Beagle dogs. Consequently, the NOAEL was determined to be 5 mg/kg bw/day in male and female Beagle dogs.

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

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