The Japanese Society of Toxicology

Fundamental Toxicological Sciences

URL : http://www.fundtoxicolsci.org/index_e.html

Original Article

Safety evaluation of tomatidine-rich tomato leaf extract in mice and bacteria

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(Received July 3, 2024; Accepted July 12, 2024)

ABSTRACT — Tomatidine is an aglycone of α-tomatine, a glycoalkaloid present in tomato plants, and has muscle atrophy inhibitory effect and anticancer activity. Tomatidine-Rich Tomato Leaf Extract powder (TRTLE) contains 60% tomatidine, which is converted to tomatidine by acid hydrolysis after extracting α-tomatine from tomato leaves. The purpose of this study was to evaluate the safety of TRTLE by conducting a series of toxicity studies in mice and bacteria to support its safe food use. Single-dose and 90-day repeated-dose toxicity study were conducted in 6-week-old ICR male and female mice to calculate the LD_{50} and non-toxic dose (NOAEL) of TRTLE. In the single-dose toxicity study, a single oral dose of 667, 2,000, and 5,000 mg TRTLE/kg body weight (bw) was administered, and in the 90-day repeated-dose toxicity study, 133 mg TRTLE/kg bw was administered orally daily. In addition, the Ames test was performed with *Salmonella* Typhimurium and *Escherichia coli* to determine the genotoxic activity. The single-dose toxicity study indicated the LD_{50} was 833 mg TRTLE/kg bw (tomatidine equivalent: 500 mg/kg bw). In the 90-day repeated-dose toxicity study, no abnormalities due to TRTLE were observed in each laboratory test, including general symptoms, body weight changes, hematology, urinalysis, and histopathological examination. In the Ames test, TRTLE was confirmed not to be mutagenic with or without metabolic activation. Based on these data, the NOAEL in mice was determined to be 133 mg TRTLE/kg bw (tomatidine equivalent: 80 mg/kg bw).

Key words: α-Tomatine, Tomatidine, Tomato leaf extract, Single-dose toxicity study, 90-day repeated-dose toxicity study, Ames test

INTRODUCTION

A Tomato (Lycopersicon esculentum Mill.) is one of the most widely grown and consumed vegetable crops in the world and is a familiar source of nutrition to people worldwide (Hussein *et al*., 2016; Reimers and Keast, 2016). The glycoalkaloid α-tomatine, which is abundant in unripe fruits (green tomatoes), is thought to function as a protective and repellent component against plant predators such as fungi, bacteria, viruses, and insects (Friedman, 2002; Bailly, 2021). Its physiological activity has been reported to inhibit cancer cell proliferation and bone loss (Friedman *et al*., 2009; Nirmala *et al*., 2020). Tomatidine is an aglycone of α-tomatine and has been reported to function as an inhibitor muscle atrophy (Dyle *et al*., 2014; Ebert *et al*., 2015), anti-cancer effects (Fujimaki *et al*., 2022), and life span extension effect in *C. elegans* (Fang *et al*., 2017).

Glycoalkaloids are at risk of causing food poisoning (gastrointestinal symptoms such as nausea, vomiting, and diarrhea) if ingested by humans (Mensinga *et al*., 2005), and there is concern that α-tomatine is also toxic to humans. However, Lycopersicon esculentum var. cerasiforme (a tomato variety grown in Peru)

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is widely consumed locally without apparent acute toxic effects, despite its high concentration of α-tomatine (500–5,000 mg/kg dry weight) (Friedman, 2002). Also, α-tomatine is considered to be less toxic when hydrolyzed to tomatidine (Arneson and Durbin, 1968; Sandrock and VanEtten, 1998). This is related to the structure of tomatidine; it is presumably due to the absence of the 5,6-double bond in the B ring of the steroid skeleton (Friedman *et al*., 2003). However, little is known about the toxicity of tomatidine, and there are many unknowns about the risk of adverse health effects (Schrenk *et al*., 2020).

α-Tomatine is abundant in tomato unripe fruits and even more so in leaves (Friedman, 2002). Tomatoes are widely produced around the world, but their leaves produced during the cultivation process are considered nothing more than industrial waste. We attempted to effectively utilize tomato leaves, an underutilized resource, as a source of tomatidine. We extracted α-tomatine from tomato leaves, followed by acid hydrolysis to obtain a Tomatidine-Rich Tomato Leaf Extract powder (TRTLE). The production of TRTLE contributes to the development of a recycling society in that it converts waste into a functional material (tomatidine). The effects of TRTLE have already been reported to inhibit tumor growth *in vivo* and the proliferation of 85As2 cells derived from human gastric cancer *in vitro* (Fujimaki *et al*., 2022). However, since there is generally no experience in eating tomato leaves, it was necessary to conduct toxicity tests to assure the safety of TRTLE for use as an edible. Here we show the results of safety evaluation of TRTLE in a single-dose toxicity study and 90-day repeated-dose study in mice, as well as in an Ames study in *Salmonella* Typhimurium (*S*. Typhimurium) and *Escherichia coli* (*E. coli*).

MATERIALS AND METHODS

Preparation of TRTLE

TRTLE was prepared in the same way as previously reported (Fujimaki *et al*., 2022; Suzuki *et al*., 2022). Tomato leaves, waste material generated in the process of tomato cultivation, were provided by Smart Agriculture IWATA Co., Ltd. (Shizuoka, Japan), and then hot air-dried at Mizuno Foods Co., Ltd. (Shizuoka, Japan). To dried tomato leaves, 15 volumes (by weight) of water were added, adjusted to pH 3.5 with hydrochloric acid, and then extracted at 80°C for 30 min. The extract was clarified by diatomaceous earth filtration to obtain an extract solution containing α-tomatine. Then, trisodium citrate dihydrate was added as a chelating agent to a concentration of 4% (w/w), and sodium hydroxide solution was added to adjust the pH to 8.5. α-Tomatine was then precipitated and deposited. The precipitate containing α-tomatine was collected by centrifugation (4,000 × *g*, 10 min). Then, α-tomatine was solubilized by adding hydrochloric acid to the precipitate and adjusting the hydrochloric acid concentration to 1.3 N, and then converted to tomatidine by heating at 80°C for 2 hr. Tomatidine was precipitated by adjusting the solution to pH 8.5 by adding sodium hydroxide; the resulting tomatidine-containing precipitate was collected by centrifugation (4,000 × *g*, 10 min), and the precipitate was lyophilized to obtain TRTLE. As confirmed an HPLC analysis method (Taveira *et al*., 2012), this TRTLE contained 60% tomatidine and neither α-tomatine nor dehydrotomatine was detected.

Compliance in mice toxicity studies

All animal investigations were conducted in accordance with the "Law Concerning the Protection and Management of Animals" (Law No. 46, May 30, 2014) and the "Standards for the Care and Keeping of Laboratory Animals and the Reduction of Pain" (Ministry of the Environment Notification No. 84, August 30, 2013).

Single-dose toxicity study

Tests were conducted at the Japan Food Research Laboratories (Tama Laboratory, Tokyo, Japan) in accordance with the "OECD Guideline for Testing of Chemicals 420" (OECD, 2001) and the ethics criteria contained in the bylaw of the Committee of the Japan Food Research Laboratories.

The test animals were 5-week-old male and female ICR mice purchased from Japan SLC Inc. (Shizuoka, Japan) and acclimated for 1 week. The mice were housed in polycarbonate cages (five mice per cage) at a room temperature of 23°C ± 3°C under a 12-hr light/dark cycle, and fed ad libitum on Lab MR stock (Nosan Corporation, Kanagawa, Japan) and tap water.

The test substance, TRTLE, was suspended in water for injection to prepare solutions of 33.35, 100, and 250 mg/mL. TRTLE was administered at doses of 667, 2,000, and 5,000 mg/kg body weight (bw); the control group received water for injection as a solvent control. Each group consisted of five male and five female mice. Prior to administration, test animals were fasted for approximately 4 hr, weighed, and then administered single doses of test solution (to the TRTLE-treated group) or water for injection (to the control group) at a dosage volume of 20 mL/kg bw using a gastric sonde. The observation period was 14 days, with frequent observations on the day of administration and then subsequently once

per day. Body weight was measured 7 and 14 days after administration. At the end of the observation period, all mice (whether they died or survived) were autopsied.

90-day repeated-dose toxicity study

(a) Animals and test substance administration

Tests were approved by the Institutional Animal Care and Use Committee and conducted at the BioSafety Research Center Inc. (Shizuoka, Japan) and in accordance with the "OECD test guideline 408" (OECD, 1998) and the ethics criteria contained in the bylaw of the Committee of the Biosafety Research Center.

Test animals were 5-week-old male and female ICR mice purchased from Charles River Laboratories Japan Inc. (Kanagawa, Japan) and acclimated for 1 week. One animal at a time was housed in a metal tethered rearing cage (W 10.0 × D 19.6 × H 13.0 cm) and fed ad libitum on radiation-sterilized solid feed CRF-1 (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water.

The rearing cages were changed once every other week and the feeders were changed once a week. The dose of TRTLE was set at 133 mg/kg bw (tomatidine equivalent: 80 mg/kg bw). The dose was set equivalent to 100 times higher than daily intake, based on the assumptions that the expected intake of tomatidine in humans is 40 mg/day and the average body weight of Japanese is 50 kg. TRTLE was mixed with 0.5 w/v% CMC-Na solution to prepare a test solution of 13.3 mg/mL, which was administered to mice of 11 males and 11 females per group by forced oral gavage once a day for 90 days. The dose volume was 0.1 mL per 10 g of body weight, and was calculated based on the more recent body weight measurement for each individual. After 90 days, six male and six female mice/group were euthanized and dissected; for the remaining five male and five female mice/group, a 28-day recovery test was established to evaluate the delayed, persistent, and reversible effects of test substance administration.

(b) Observation, measurement, and examination

General condition

All animals were observed twice daily for mortality, morbidity, appearance, and behavioral abnormalities. During the recovery period, observations were made once daily.

Body weight and food consumption

Body weight was measured once a week during the study period prior to administration of the test substance. The weight gain was calculated as the change in body weight from the first day of the study (Day 1) to the last day of the study (Day 90), or from the first day of the recovery period (Day 91) to the last day (Day 118). Food consumption was measured once a week, and the average daily food intake (g/day) was calculated from the difference in food weight between the days of measurement.

Hematology and blood chemistry

Mice were laparotomized under isoflurane anesthesia, and blood was collected from the abdominal aorta. Blood samples were collected in anticoagulant (EDTA-2K)-containing blood collection tubes (BD Microtina® MAP micro blood collection tubes, Becton Dickinson Company, Ltd., Tokyo, Japan) for hematological examination and were measured on a comprehensive hematology analyzer (ADVIAl20, Bayer Yakuhin, Ltd., Osaka, Japan). Tests included hematocrit (HCT), hemoglobin (HGB), red blood cell (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte, platelet (PLT), white blood cell (WBC), neutrophil (NEUT), lymphocyte (LYMPH), monocyte (MONO), eosinophil (EOSN), basophil (BASO) and large unstained cell (LUC). Blood chemistry tests were performed using a blood collection tube (BD Microtina® micro blood collection tube, BD Microguard™, Becton Dickinson) containing an anticoagulant (lithium phosphate). Plasma obtained by centrifugation of blood at 1700 × *g* for 15 min at room temperature was analyzed using a multiparameter automated biochemical analyzer (Hitachi 7170, Hitachi Ltd., Tokyo, Japan) and a fully automated electrolyte analyzer (EAO7, A&T Corporation, Kanagawa, Japan). Tests included total protein (TP), albumin, globulin, albumin/globulin ratio (A/G ratio), glucose, Triglyceride, total cholesterol (T-Chol), blood urea nitrogen (BUN), creatinine, total bilirubin (T-Bili), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), calcium, inorganic phosphorus (IP), sodium, potassium and chloride.

Urinalysis

Urine was collected by a urine collector for 24 hr under the feeding and watering conditions used. The collection periods were 3 days before the last day of the toxicity and the recovery test, respectively (Days 87 and 115). After examination of urine volume and specific gravity, urinary electrolyte (sodium, potassium, and chloride) concentrations and total excretion were calculated using the supernatant obtained after the urine was centrifuged at approximately 400 × *g* for 5 min and separated.

Necropsy

After blood samples were drawn from the aorta under isoflurane anesthesia, the mice were euthanized by blood loss. Gross necropsy of the brain, heart, lungs (including bronchi), liver (including gallbladder), kidneys, adrenal glands, testes, ovaries, thymus, and spleen was performed and organ weights were determined. The organ weight ratio (relative weight) was calculated from the body weight and organ weight on the day of necropsy ((organ weight/final body weight) × 100). For histopathological examination, histopathology specimens were prepared for the liver of all animals and for organs and tissues with gross abnormalities. After tissues were fixed in 10% neutral buffered formalin solution, paraffin-embedded sections were prepared and stained with hematoxylin–eosin (H.E.). The specimens were examined and the findings, including the type and extent of lesions, were entered directly into a computer using a pathology system (PATHOTOX).

(c) Statistical analysis

For quantitative values (body weight, body weight gain, food intake, hematology, blood biochemistry, urinalysis, organ weight, and relative weight), the mean and standard deviation for each group were calculated, and a test of equal variance (F-test) was performed between the control and test substance treatment groups at a two-tailed 5% significance level. The statistical significance tests performed were Student's t-test for equal variances and Aspin–Welch's t-test for unequal variances, with two-tailed significance levels of 5% and 1%. No statistical analysis was performed on the general condition, autopsy, or histopathological examination results.

Ames test

The study was conducted at BML Inc. (BML General Laboratory, Saitama, Japan) under contract with New Drug Research Center Inc. (Hokkaido, Japan) in accordance with the "Guidance on Genotoxicity Testing and Interpretation of Drugs" (September 20, 2012, PFSB/ELD Notification No. 0920, No. 2) and "OECD test guideline 471" (OECD, 1997).

In the Ames test, histidine-dependent nutrient-requiring mutants of *S.* Typhimurium TA100, TA1535, TA98, and TA1537, and tryptophan-dependent nutrient-requiring mutants of *E. coli* WP2*uvr*A, a tryptophan-dependent nutrient-requiring mutant of *E. coli* WP2 (Ames *et al*., 1973; McCann *et al*., 1975; Green and Muriel, 1976) were exposed to the test compound and its mutagenic potential was determined. *S.* Typhimurium TA100 strain was obtained from the Mutagenicity Division of the National Institutes of Health. Other *S.* Typhimurium TA strains were obtained from Prof. Ames, U.C. Berkeley. *E. coli* WP2*uvr*A was obtained from the Institute of Cancer Biology, Institute of Medical Science, University of Tokyo.

TRTLE was suspended in DMSO and a dose-finding study was conducted to establish the dose for this study. The dose-finding study tested seven different concentrations of test substance (1.2, 4.9, 20, 78, 313, 1,250, and 5,000 μg/plate) in all strains of murine typhoid and *E. coli*, and in the presence and absence of metabolic S9 activation.

Confirmatory tests were performed by preincubation method (Yahagi *et al*., 1977; Maron and Ames, 1983). The number of reversion mutant colonies was measured visually because precipitation of the test material was observed on the plates. Only the positive control was measured with an automatic colony counter CA-11 (System Science Co., Ltd., Tokyo, Japan). As a rule, results were judged positive when the number of reversion mutant colonies in the TRTLE-treated group was significantly increased (twice that of the negative control) relative to the number of spontaneous reversion mutant colonies, and when dose–response and reproducibility were observed. No particular statistical treatment was used in the analysis of the genotoxicity.

RESULTS

Single-dose toxicity study

In the 667 mg TRTLE/kg bw group, there were no deaths in males during the observation period, but two deaths were observed in females within 1 day of administration (mortality rate: 40%). In the 2,000, 5,000 mg TRTLE/kg bw groups, all mice died within 1 day after administration in males and within 3 days in females (100% mortality). Observations of the general condition of the 667 mg TRTLE/kg bw group revealed decreased spontaneous locomotion and abnormal body posture within a few hours after administration, but these symptoms recovered within 1 day after administration in surviving animals, and body weights at the end of the 14-day observation period were not significantly different from those in the control group. No abnormalities were observed in major organs at autopsy. The LD_{50} values, calculated from the number of dead animals killed in each group on the 14th day after administration, were 1,330 mg TRTLE/kg bw for males (tomatidine equivalent: 800 mg/kg bw) and 830 mg TRTLE/kg bw for females (tomatidine equivalent: 500 mg/kg bw).

90-day repeated-dose toxicity study

(a) General condition

There were no deaths in males or females during the administration or recovery period, and no abnormalities in general condition that could be attributed to the administration of the test substance.

(b) Body weight

There were no statistically significant changes in the body weight of male or female mice during treatment with TRTLE compared to the control group (Fig. 1). There was also no significant change in body weight gain during the treatment period. During the recovery period, the amount of body weight gain was significantly lower in the TRTLE-treated male group (Table 1).

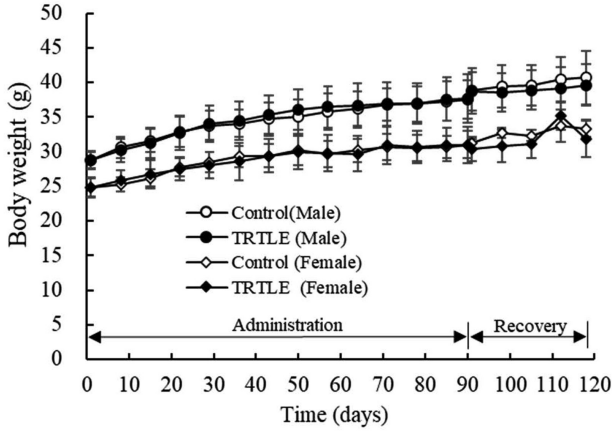

Fig. 1. Body weight changes in male and female mice administered TRTLE for 90 days. Numbers indicate mean ± S.D., (administration: n = 11; recovery: n = 5).

(c) Food consumption

The mean daily food intake of males in the TRTLE-treated group for Days 29–36 and 36–43 of the treatment period and the mean daily food intake of females in the TRTLE-treated group for Days 50–57, 57–64, 64–71, and 71–78 were statistically significantly higher than those in the control group (Fig. 2). During the recovery period, females in the TRTLE-treated group had statistically significantly higher mean daily food intake on Days 91–98 compared with the control group.

(d) Hematology

At the end of the treatment period, there were no statistically significant changes in any of the laboratory parameters in the male and female test animals in the TRTLE-treated group with the control group (Table 2). At the end of the recovery period, WBC count, LYMPH count, EOSN count and ratio were statistically significantly lower in females in the TRTLE-treated group compared with the control group.

Table 1. The body weight gain in male and female mice administered TRTLE for 90 days.

		Gain (g)	
		Administration	Recovery
Male	Control	8.7 ± 2.6	2.0 ± 0 .9
	TRTLE	8.9 ± 2.8	0.8 ± 0.3*
Female	Control	6.2 ± 1.3	2.0 ± 1.4
	TRTLE	6.2 ± 2.8	1.5 ± 1.0

Numbers indicate mean ± S.D., * P < 0.05 (Student's *t*-test).

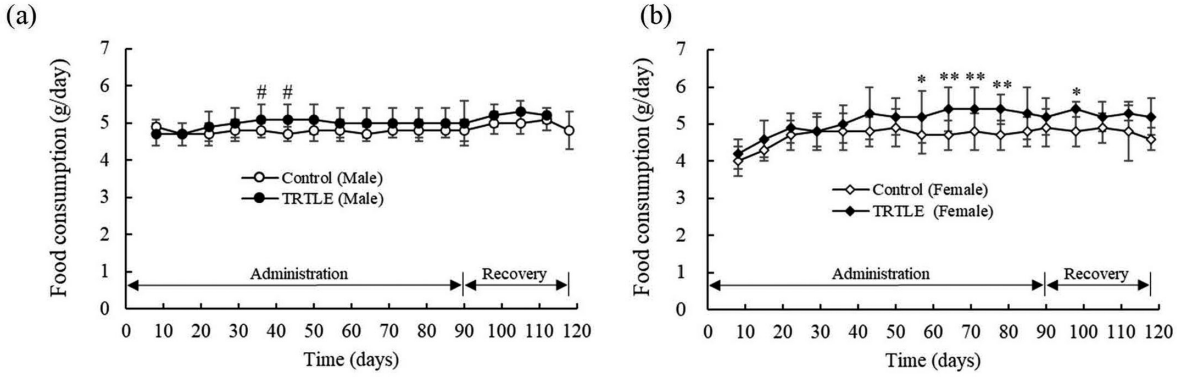

Fig. 2. Food consumption changes for male (a) and female mice (b) administered TRTLE for 90 days. Numbers indicate mean ± S.D., (administration: n = 11, recovery: n = 5), # P < 0.05 (Welch–Aspin test), * and ** P < 0.05 and P < 0.01 (Student's *t*-test).

Table 2. Hematology values for male and female mice administered TRTLE for 90 days.

		Male		Female	
		Control	TRTLE	Control	TRTLE
HCT (%)		42.3 ± 1.5	41.8 ± 1.8	41.7 ± 1.7	42.2 ± 1.9
HGB (g/dL)		13.5 ± 0.4	13.4 ± 0.6	13.4 ± 0.4	13.8 ± 0.6
RBC ($\times 10^6$/mm^3)		8.63 ± 0.46	8.64 ± 0.32	8.55 ± 0.47	8.57 ± 0.50
MCV (μm^3)		49.1 ± 1.0	48.4 ± 1.3	48.8 ± 1.7	49.3 ± 1.6
MCH (pg)		15.6 ± 0.5	15.5 ± 0.4	15.7 ± 0.7	16.1 ± 0.6
MCHC (%)		31.8 ± 0.7	32.0 ± 0.4	32.2 ± 0.7	32.7 ± 0.7
Reticulocyte (%)		3.4 ± 0.3	3.3 ± 0.2	4.3 ± 1.2	3.3 ± 0.5
Reticulocyte ($\times 10^9$/L)		292.3 ± 25.4	285.9 ± 21.1	368.3 ± 109.3	284.6 ± 30.7
PLT ($\times 10^3$/mm^3)		1321 ± 58	1368 ± 244	1332 ± 274	1178 ± 160
WBC ($\times 10^3$/mm^3)		2.96 ± 0.43	2.91 ± 1.04	1.62 ± 0.69	1.53 ± 0.99
	NEUT (%)	18.5 ± 2.7	19.3 ± 4.1	17.9 ± 9.6	16.1 ± 4.9
	LYMPH (%)	76.6 ± 3.9	74.8 ± 4.5	75.9 ± 10.0	77.0 ± 4.0
Differential	MONO (%)	2.2 ± 0.7	2.1 ± 0.3	1.7 ± 0.7	2.1 ± 1.0
leukocyte rations	EOSN (%)	2.4 ± 1.3	3.6 ± 1.1	4.1 ± 1.6	4.7 ± 2.6
	BASO (%)	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
	LUC (%)	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.3	0.3 ± 0.1
NEUT ($\times 10^3$/mm^3)		0.55 ± 0.11	0.55 ± 0.21	0.30 ± 0.25	0.23 ± 0.14
LYMPH ($\times 10^3$/mm^3)		2.27 ± 0.34	2.18 ± 0.81	1.22 ± 0.57	1.20 ± 0.82
MONO ($\times 10^3$/mm^3)		0.07 ± 0.03	0.06 ± 0.02	0.03 ± 0.02	0.03 ± 0.03
EOSN ($\times 10^3$/mm^3)		0.07 ± 0.04	0.10 ± 0.06	0.06 ± 0.03	0.07 ± 0.03
BASO ($\times 10^3$/mm^3)		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
LUC ($\times 10^3$/mm^3)		0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00
(Recovery group)					
HCT (%)		40.8 ± 3.3	40.7 ± 2.0	40.9 ± 1.5	41.2 ± 0.8
HGB (g/dL)		13.2 ± 0.7	13.2 ± 0.5	13.3 ± 0.5	13.5 ± 0.4
RBC ($\times 10^6$/mm^3)		8.57 ± 0.71	8.69 ± 0.53	8.74 ± 0.61	8.60 ± 0.20
MCV (μm^3)		47.7 ± 1.1	46.9 ± 1.6	46.9 ± 2.1	47.9 ± 1.8
MCH (pg)		15.4 ± 0.7	15.2 ± 0.4	15.3 ± 0.6	15.7 ± 0.7
MCHC (%)		32.5 ± 1.3	32.4 ± 0.7	32.6 ± 0.4	32.7 ± 0.7
Reticulocyte (%)		3.3 ± 0.3	3.5 ± 0.6	3.8 ± 0.8	3.4 ± 0.6
Reticulocyte ($\times 10^9$/L)		279.2 ± 21.8	305.7 ± 47.5	336.5 ± 82.0	291.3 ± 48.6
PLT ($\times 10^3$/mm^3)		1415 ± 176	1385 ± 87	1178 ± 64	1185 ± 137
WBC ($\times 10^3$/mm^3)		2.09 ± 0.98	2.65 ± 1.71	1.86 ± 0.61	1.00 ± 0.31*
	NEUT (%)	29.6 ± 10.4	17.9 ± 8.3	23.0 ± 11.6	20.4 ± 7.8
	LYMPH (%)	61.2 ± 10.4	75.8 ± 8.0	68.0 ± 12.7	75.5 ± 8.3
Differential	MONO (%)	3.1 ± 1.2	2.0 ± 0.1	2.0 ± 0.9	1.2 ± 0.4
leukocyte rations	EOSN (%)	5.7 ± 1.3	3.9 ± 2.7	6.7 ± 3.2	2.6 ± 0.9#
	BASO (%)	0.0 ± 0.1	0.1 ± 0.1	0.0 ± 0.1	0.1 ± 0.1
	LUC (%)	0.4 ± 0.3	0.3 ± 0.1	0.2 ± 0.2	0.1 ± 0.1
NEUT ($\times 10^3$/mm^3)		0.61 ± 0.35	0.35 ± 0.16	0.46 ± 0.39	0.21 ± 0.12
LYMPH ($\times 10^3$/mm^3)		1.28 ± 0.61	1.96 ± 1.65	1.23 ± 0.33	0.75 ± 0.22*
MONO ($\times 10^3$/mm^3)		0.06 ± 0.03	0.05 ± 0.04	0.04 ± 0.02	0.01 ± 0.00
EOSN ($\times 10^3$/mm^3)		0.12 ± 0.07	0.09 ± 0.08	0.12 ± 0.07	0.03 ± 0.01#
BASO ($\times 10^3$/mm^3)		0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
LUC ($\times 10^3$/mm^3)		0.01 ± 0.01	0.01 ± 0.02	0.00 ± 0.00	0.00 ± 0.00

Numbers indicate mean ± S.D., * $P < 0.05$ (Student's *t*-test), # $P < 0.05$ (Welch–Aspin test).

Table 3. Blood biochemistry parameters values for male and female mice administered TRTLE for 90 days.

	Male		Female	
	Control	TRTLE	Control	TRTLE
TP (g/dL)	4.98 ± 0.17	5.02 ± 0.20	5.01 ± 0.15	4.92 ± 0.20
Albumin (g/dL)	2.98 ± 0.11	2.88 ± 0.19	2.97 ± 0.21	3.13 ± 0.16
Globulin (g/dL)	2.01 ± 0.09	2.15 ± 0.11*	2.04 ± 0.27	1.80 ± 0.09
A/G ratio	1.49 ± 0.07	1.35 ± 0.12*	1.49 ± 0.25	1.75 ± 0.11*
Glucose (mg/dL)	260 ± 13	260 ± 17	237 ± 21	228 ± 17
Triglyceride (mg/dL)	96 ± 38	147 ± 65	79 ± 32	75 ± 27
T-Chol (mg/dL)	134 ± 20	164 ± 39	105 ± 20	106 ± 14
BUN (mg/dL)	31.2 ± 5.6	35.8 ± 4.2	21.6 ± 1.4	23.6 ± 3.6
Creatinine (mg/dL)	0.10 ± 0.03	0.11 ± 0.02	0.09 ± 0.01	0.09 ± 0.02
T-Bili (mg/dL)	0.09 ± 0.02	0.06 ± 0.02*	0.05 ± 0.02	0.05 ± 0.01
AST (U/L)	55 ± 33	40 ± 13	49 ± 8	46 ± 6
ALT (U/L)	44 ± 28	28 ± 7	24 ± 5	26 ± 6
ALP (U/L)	154 ± 24	181 ± 59	257 ± 80	228 ± 62
Calcium (mg/dL)	9.12 ± 0.32	9.26 ± 0.28	9.08 ± 0.22	8.97 ± 0.11
IP (mg/dL)	4.96 ± 1.26	4.50 ± 1.07	4.39 ± 0.7	5.65 ± 0.68**
Sodium (mmol/L)	148.1 ± 0.8	148.6 ± 1.7	149.4 ± 1.1	150.0 ± 1.6
Potassium (mmol/L)	4.15 ± 0.37	4.64 ± 0.35*	3.56 ± 0.08	3.66 ± 0.25
Chloride (mmol/L)	111.5 ± 2.1	112.5 ± 1.7	116.8 ± 1.2	117.5 ± 1.3
(Recovery group)				
TP (g/dL)	4.99 ± 0.15	5.08 ± 0.20	4.93 ± 0.16	4.79 ± 0.16
Albumin (g/dL)	2.99 ± 0.21	2.99 ± 0.12	3.13 ± 0.12	3.06 ± 0.11
Globulin (g/dL)	2.00 ± 0.08	2.08 ± 0.13	1.80 ± 0.08	1.72 ± 0.08
A/G ratio	1.50 ± 0.16	1.44 ± 0.10	1.75 ± 0.09	1.78 ± 0.10
Glucose (mg/dL)	260 ± 28	259 ± 39	229 ± 12	216 ± 56
Triglyceride (mg/dL)	158 ± 47	106 ± 63	61 ± 23	62 ± 13
T-Chol (mg/dL)	135 ± 23	155 ± 22	93 ± 12	93 ± 18
BUN (mg/dL)	38.2 ± 9.8	32.4 ± 3.3	20.3 ± 2.3	23.5 ± 3.7
Creatinine (mg/dL)	0.09 ± 0.02	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.02
T-Bili (mg/dL)	0.07 ± 0.03	0.07 ± 0.01	0.09 ± 0.03	0.07 ± 0.01
AST (U/L)	48 ± 7	56 ± 19	43 ± 4	50 ± 5*
ALT (U/L)	39 ± 11	38 ± 7	24 ± 4	23 ± 4
ALP (U/L)	149 ± 25	166 ± 31	227 ± 72	233 ± 25
Calcium (mg/dL)	8.99 ± 0.22	8.94 ± 0.37	9.08 ± 0.27	8.87 ± 0.36
IP (mg/dL)	3.96 ± 0.98	4.16 ± 0.96	5.87 ± 1.26	5.48 ± 0.87
Sodium (mmol/L)	150.6 ± 2.1	151.1 ± 2.7	150.3 ± 1.6	150.7 ± 1.1
Potassium (mmol/L)	4.55 ± 0.16	4.25 ± 0.52	4.14 ± 0.33	3.87 ± 0.23
Chloride (mmol/L)	115.3 ± 3.5	114.9 ± 2.6	117.2 ± 2.3	119.1 ± 2.2

Numbers indicate mean ± S.D., * and ** $P < 0.05$ and $P < 0.01$ (Student's *t*-test).

(e) Blood biochemistry

At the end of the treatment period, males in the TRTLE-treated group had statistically significantly higher levels of globulin and potassium and significantly lower A/G ratio and T-Bili than controls (Table 3). A/G ratio and IP levels were significantly higher in the TRTLE-treated females. At the end of the recovery period, AST was statistically significantly higher in females in the TRTLE-treated group compared with the control group.

(f) Urinalysis

At the end of the treatment period, specific gravity, sodium, potassium, and chloride concentrations were statistically significantly higher in females in the TRTLE-treated group than the control group (Table 4); no significant changes were observed for males. At the end of the recovery period, there were no significant changes in any of the test parameters in males and females in the TRTLE-treated group compared with the control group.

Table 4. Urinalysis parameter values for male and female mice administered TRTLE for 90 days.

	Male		Female	
	Control	TRTLE	Control	TRTLE
Volume (mL)	1.5 ± 0.7	1.6 ± 0.4	2.0 ± 0.4	1.5 ± 0.6
Specific gravity	1.076 ± 0.011	1.075 ± 0.016	1.055 ± 0.008	1.081 ± 0.020*
Sodium (mmol/L)	159.7 ± 24.0	157.2 ± 44.0	111.4 ± 28.2	166.6 ± 37.9*
Potassium (mol/L)	311.0 ± 42.9	300.0 ± 81.7	206.1 ± 32.8	328.4 ± 78.0**
Chloride (mol/L)	232.9 ± 37.3	233.3 ± 63.3	170.8 ± 29.8	261.5 ± 65.7*
Sodium (mmol/day)	0.23 ± 0.08	0.24 ± 0.05	0.21 ± 0.02	0.24 ± 0.06
Potassium (mmol/day)	0.45 ± 0.17	0.47 ± 0.13	0.39 ± 0.04	0.46 ± 0.08
Chloride (mmol/day)	0.34 ± 0.11	0.36 ± 0.09	0.32 ± 0.02	0.36 ± 0.05
(Recovery group)				
Volume (mL)	1.5 ± 0.5	1.3 ± 0.9	1.8 ± 1.3	1.3 ± 0.7
Specific gravity	1.072 ± 0.014	1.071 ± 0.026	1.076 ± 0.031	1.086 ± 0.028
Sodium (mmol/L)	147.3 ± 32.0	144.5 ± 44.2	140.8 ± 35.7	173.6 ± 35.9
Potassium (mol/L)	277.7 ± 58.8	274.4 ± 127.5	285.4 ± 95.8	347.7 ± 121.8
Chloride (mol/L)	213.2 ± 46.5	209.7 ± 100.1	221.1 ± 66.4	266.5 ± 74.8
Sodium (mmol/day)	0.22 ± 0.05	0.18 ± 0.09	0.23 ± 0.11	0.21 ± 0.08
Potassium (mmol/day)	0.41 ± 0.09	0.33 ± 0.18	0.44 ± 0.20	0.1 ± 0.10
Chloride (mmol/day)	0.32 ± 0.08	0.25 ± 0.12	0.34 ± 0.15	0.32 ± 0.09

Numbers indicate mean ± S.D., * and ** $P < 0.05$ and $P < 0.01$ (Student's *t*-test).

Table 5. Organ weights for male and female mice administered TRTLE for 90 days.

	Male		Female	
	Control	TRTLE	Control	TRTLE
Body weight (g)	36.4 ± 2.3	37.3 ± 4.2	30.5 ± 2.6	31.7 ± 3.6
Brain (g)	0.53 ± 0.03	0.55 ± 0.02	0.57 ± 0.02	0.56 ± 0.02
Heart (g)	0.17 ± 0.01	0.18 ± 0.02	0.16 ± 0.01	0.16 ± 0.02
Lungs (g)	0.22 ± 0.01	0.23 ± 0.02	0.22 ± 0.02	0.24 ± 0.02
Liver (g)	2.09 ± 0.09	2.37 ± 0.27	1.74 ± 0.19	1.87 ± 0.24
Kidneys (g)	0.57 ± 0.04	0.61 ± 0.12	0.42 ± 0.04	0.43 ± 0.05
Adrenal glands (mg)	5 ± 1	5 ± 1	10 ± 1	12 ± 3
Testes (g)	0.25 ± 0.04	0.27 ± 0.02	-	-
Ovaries (g)	-	-	18 ± 3	24 ± 9
Thymus (mg)	33 ± 9	36 ± 10	37 ± 9	42 ± 11
Spleen (mg)	102 ± 21	101 ± 16	140 ± 34	112 ± 18
(Recovery group)				
Body weight (g)	40.7 ± 3.7	39.2 ± 3.5	33.0 ± 1.3	31.4 ± 2.1
Brain (g)	0.55 ± 0.02	0.57 ± 0.02	0.57 ± 0.03	0.55 ± 0.02
Heart (g)	0.19 ± 0.02	0.18 ± 0.01	0.16 ± 0.01	0.16 ± 0.02
Lungs (g)	0.22 ± 0.02	0.24 ± 0.03	0.23 ± 0.02	0.23 ± 0.01
Liver (g)	2.36 ± 0.29	2.37 ± 0.44	1.78 ± 0.15	1.65 ± 0.32
Kidneys (g)	0.65 ± 0.09	0.65 ± 0.04	0.43 ± 0.02	0.47 ± 0.04
Adrenal glands (mg)	4 ± 1	5 ± 2	8 ± 2	10 ± 2
Testes (g)	0.26 ± 0.02	0.31 ± 0.07	-	-
Ovaries (g)	-	-	20 ± 5	22 ± 5
Thymus (mg)	31 ± 7	29 ± 7	32 ± 8	30 ± 5
Spleen (mg)	114 ± 24	116 ± 36	121 ± 11	126 ± 23

Numbers indicate mean ± S.D., -: not applicable.

Table 6. Relative organ weight values for male and female mice administered TRTLE for 90 days.

	Male		Female	
	Control	TRTLE	Control	TRTLE
Brain (%)	1.452 ± 0.070	1.496 ± 0.159	1.876 ± 0.180	1.790 ± 0.172
Heart (%)	0.473 ± 0.048	0.487 ± 0.026	0.538 ± 0.036	0.519 ± 0.057
Lungs (%)	0.615 ± 0.055	0.618 ± 0.024	0.723 ± 0.032	0.746 ± 0.064
Liver (%)	5.748 ± 0.304	6.355 ± 0.504*	5.693 ± 0.424	5.901 ± 0.261
Kidneys (%)	1.561 ± 0.154	1.611 ± 0.140	1.385 ± 0.114	1.374 ± 0.143
Adrenal glands (%)	0.014 ± 0.003	0.013 ± 0.004	0.032 ± 0.004	0.036 ± 0.009
Testes (%)	0.691 ± 0.097	0.733 ± 0.066	-	-
Ovaries (%)	-	-	0.059 ± 0.010	0.074 ± 0.027
Thymus (%)	0.089 ± 0.023	0.098 ± 0.032	0.120 ± 0.029	0.133 ± 0.025
Spleen (%)	0.277 ± 0.044	0.273 ± 0.049	0.462 ± 0.122	0.352 ± 0.043
(Recovery group)				
Brain (%)	1.345 ± 0.071	1.455 ± 0.106	1.738 ± 0.127	1.770 ± 0.134
Heart (%)	0.456 ± 0.015	0.465 ± 0.018	0.486 ± 0.031	0.523 ± 0.055
Lungs (%)	0.543 ± 0.066	0.611 ± 0.017	0.711 ± 0.073	0.734 ± 0.063
Liver (%)	5.797 ± 0.533	6.008 ± 0.708	5.405 ± 0.308	5.218 ± 0.703
Kidneys (%)	1.597 ± 0.129	1.665 ± 0.052	1.318 ± 0.087	1.497 ± 0.080**
Adrenal glands (%)	0.011 ± 0.003	0.013 ± 0.005	0.026 ± 0.005	0.033 ± 0.009
Testes (%)	0.645 ± 0.051	0.790 ± 0.129*	-	-
Ovaries (%)	-	-	0.061 ± 0.017	0.071 ± 0.012
Thymus (%)	0.077 ± 0.022	0.074 ± 0.018	0.098 ± 0.023	0.097 ± 0.018
Spleen (%)	0.282 ± 0.063	0.293 ± 0.070	0.368 ± 0.028	0.399 ± 0.050

Numbers indicate mean ± S.D., * and ** $P < 0.05$ and $P < 0.01$ (Student's *t*-test), -: not applicable.

(g) Organ weight

Although there were no significant differences in organ weights at the end of the treatment period (Table 5), the relative weights were significantly higher for the liver of males in the TRTLE-treated group (Table 6). At the end of the recovery period, the relative weight of the testes was significantly higher in the TRTLE-treated male group and the relative weight of the kidneys was significantly higher in the TRTLE-treated female group.

(h) Necropsy

At the end of the treatment period, no changes were observed that could be attributed to the administration of TRTLE (Table 7). In one female, findings corresponding to external genital abnormalities in the general condition were vaginal orifice closure and associated uterine and vaginal lumen dilatation and yellow contents. The other findings were either similar in number to those in the control group or occurred only one; all were considered to be spontaneous lesions often observed in mice of the same strain, and were present at the end of the recovery period.

(i) Histopathological examination

At the end of the treatment period, no findings that could be attributed to the administration of TRTLE (Table 8). In one female, a congenital abnormality of vaginal orifice closure as well as luminal dilatation with uterine and vaginal cellular remnants was observed. The other findings were either similar in number to those in the control group or occurred individually; all were considered to be spontaneous lesions frequently observed in mice of the same strain. At the end of the recovery period, there were no findings that could be attributed to the administration of TRTLE. A benign tumor, bronchioloalveolar adenoma, was observed in the lungs of one female, but it was considered to be a spontaneous tumor because it was solitary and not accompanied by associated lesions such as hyperplasia. The other findings were either similar in number to those observed in the control group or occurred individually; all were thought to be spontaneous lesions frequently observed in mice of the same strain.

Ames test

Dose-finding studies showed that TRTLE inhibited the growth of *S.* Typhimurium TA strains at ≥20 μg/plate in the absence of metabolic activation and at ≥1,250 μg/plate in the presence of metabolic activation. Precipitation of TRTLE on the plates was observed at ≥1,250 μg/plate with or without metabolic activation. Therefore, to confirm dose–response, six doses (up to a maximum vol-

Table 7. Necropsy findings for male and female mice administered TRTLE for 90 days.

Organ	Findings	Male		Female	
		Control	TRTLE	Control	TRTLE
RESPIRATORY SYSTEM					
(Recovery group)					
lungs	nodule	0	0	0	1
DIGESTIVE SYSTEM					
small intestine	adhesion	0	0	1	0
	diverticulum	0	1	0	0
	nodule	0	0	1	0
(Recovery group)					
small intestine	nodule	1	0	0	1
liver	white patch	0	0	0	1
URINARY SYSTEM					
kidneys	cyst	1	1	0	0
REPRODUCTIVE SYSTEM					
ovaries	cyst	-	-	1	2
uterus	dilated lumen	-	-	0	1
	yellow contents	-	-	0	1
vagina	atresia	-	-	0	1
	dilated lumen	-	-	0	1
	yellow contents	-	-	0	1
(Recovery group)					
ovaries	cyst	-	-	2	2
INTEGUMENTARY SYSTEM					
subcutaneous tissue	mass	0	0	1	0
SPECIAL SENSE SYSTEM					
(Recovery group)					
auricle	defect	0	0	0	1

-: not applicable.

ume of 20 μg/plate or 1,250 μg/plate) of *S.* Typhimurium TA strains without metabolic activation or with metabolic activation (except TA98), six doses of *E. coli* WP2*uvr*, five doses with a maximum dose of 5,000 μg/plate for *E. coli* WP2*uvr*A, and eight doses with a maximum dose of 5,000 μg/plate for *S.* Typhimurium TA98 with metabolic activation. The results of the preincubation test showed that treatment with TRTLE did not increase the number of reversion mutant colonies by more than two-fold compared with the negative control in either the base pair substitution or frameshifted strain, with or without metabolic activation (Fig. 3). Thus, it was confirmed that TRTLE was not mutagenic with or without metabolic activation system.

DISCUSSION

In the single-dose toxicity study, the LD_{50} value of TRTLE was calculated to be 1,330 mg/kg bw for male mice and 830 mg/kg bw for female mice, suggesting that its toxicity is greater in females than in males. Both of the toxic intensities are classified as ordinary products according to the criteria for toxic substances and deleterious substances in the Ministry of Health, Labor and Welfare's Pharmaceutical and Pharmaceutical Affairs Bureau No. 0613, No. 1. The LD_{50} values based on tomatidine content were 800 mg/kg bw for males and 500 mg/kg bw for females, which were almost the same as that previously reported for α-tomatine (LD_{50} value of 500 mg/kg bw for oral administration in mice) (Sackmann *et al*., 1959; Nishie *et al*., 1975). However, as the molecular weight of tomatidine is decreased to 40% of α-tomatine, the results of this study suggest that the toxicity of tomatidine on a molecular weight basis may be approximately 2.5 times lower than that of α-tomatine. The LD_{50} value of tomatidine has not been reported so far, many tomato plant parasites express hydrolytic enzymes, as a form of self-defense, to inhibit tomatine toxicity (Sandrock and VanEtten, 1998); tomatidine is considered to be less toxic than α-tomatine. Although we tested only TRTLE as the test substance and did not test pure tomatidine, our results were consistent with previous reports of fungal toxicity in that the toxicity per tomatidine content was lower than

Table 8. Histopathological examination findings for male and female mice administered TRTLE for 90 days.

Organ	Findings	Male		Female	
		Control	TRTLE	Control	TRTLE
REPRODUCTIVE SYSTEM					
(Recovery group)					
lungs		(0)	(0)	(0)	(0)
	adenoma, bronchiolo-alveolar	-	-	-	1
DIGESTIVE SYSTEM					
jejunum		(0)	(0)	(1)	(0)
	erosion/ulcer	-	-	1	-
ileum		(0)	(1)	(1)	(0)
	erosion/ulcer	-	0	1	-
	diverticulum	-	1	0	-
liver		(6)	(6)	(6)	(6)
	necrosis, hepatocyte	0	0	1	0
	microgranuloma	0	0	1	0
(Recovery group)					
jejunum		(1)	(0)	(0)	(0)
	erosion/ulcer	1	-	-	-
liver		(5)	(5)	(5)	(5)
	necrosis, hepatocyte	0	0	0	1
	microgranuloma	0	0	1	2
URINARY SYSTEM					
kidneys		(1)	(1)	(0)	(0)
	cyst	1	1	-	-
REPRODUCTIVE SYSTEM					
ovaries		(-)	(-)	(1)	(2)
	cyst	-	-	1	2
uterus		(-)	(-)	(0)	(1)
	dilatation, lumen	-	-	-	1
vagina		(-)	(-)	(0)	(1)
	atresia	-	-	-	1
	dilatation, lumen	-	-	-	1
(Recovery group)					
ovaries		(-)	(-)	(2)	(2)
	cyst	-	-	2	2
INTEGUMENTARY SYSTEM					
subcutaneous tissue		(0)	(0)	(1)	(0)
	inflammation	-	-	1	-
(Recovery group)					
skin		(0)	(0)	(0)	(1)
	erosion/ulcer	-	-	-	1

(): number of animal specimens, -: not applicable.

that reported for α-tomatine on a molecular weight basis.

In the 90-day repeated-dose toxicity study, significantly higher food consumption was observed in the TRTLE-treated male group, but the change was so slight that it did not affect body weight and was not judged to be due to TRTLE toxicity. Blood biochemical analysis revealed elevated levels of globulin and potassium and low levels of A/G ratio in the TRTLE-treated male group, but all changes were mild, and there were no other findings indicating inflammatory changes. In the TRTLE-treated female group had elevated A/G ratio but only slight changes in albumin and globulin. IP was increased, but no change in calcium was observed, suggesting that the changes were not indicative of impaired parathyroid or renal function. Therefore, we did not consider any of the changes to be indicative of toxic effects. In addition,

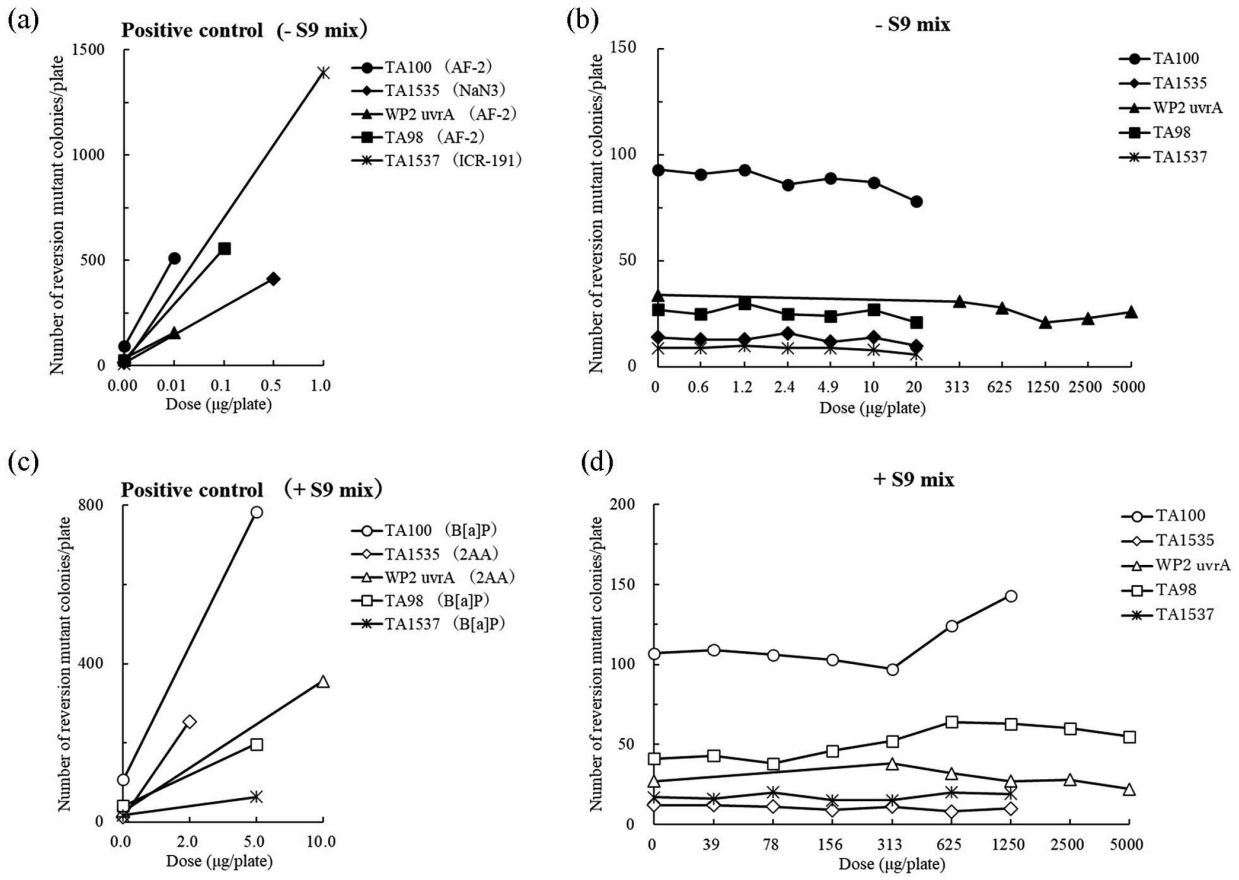

Fig. 3. Dose–response curves (− S9 mix) for positive control (a) and TRTLE (b). Dose–response curves (+ S9 mix) for positive control (c) and TRTLE (d).

T-Bili was reduced in the TRTLE-treated male group, but the change was small and judged to be of no toxicological significance. In the urinalysis, the specific gravity was higher in the TRTLE-treated female group, but the change was mild and considered to be accompanied by a nonsignificant decrease in urine volume. Sodium, potassium, and chloride concentrations were increased, but the total urinary excretion of these electrolytes was unchanged. Therefore, none of these changes were considered to indicate substance toxicity. With regard to organ weights, the relative liver weight was higher in the TRTLE-treated male group, but there was no significant difference in absolute weight, and no abnormal findings were observed on autopsy or histopathological examination; thus, we did not consider this to be due to substance toxicity.

During the 28-day recovery period and at the end of the recovery period, there were no new findings that could be related to the administration of TRTLE. Although body weight gain during the recovery period was lower in the TRTLE-treated male group, no change in body weight values was observed, and as the change was minor, it was not considered due to TRTLE toxicity. The food consumption was higher in the TRTLE-treated group, but the changes were so slight that they did not affect body weight and were not considered to be related to the administration of TRTLE. Hematology revealed low WBC count, LYMPH count, EOSN count and ratio in the TRTLE-treated female group, but as there were no changes in RBC or PLT, the changes were not considered to be due to TRTLE toxicity as they did not indicate abnormal hematopoietic function. In the blood biochemical tests, AST was elevated in the TRTLE-treated female group, but the changes were mild and were not considered to be due to TRTLE toxicity. In organ weights, in the TRTLE-treated male group had higher relative testicular weights and the TRTLE-treated female group had high-

er relative kidney weights, but the absolute weights were not significantly different, and no abnormal findings were observed at autopsy; thus, these findings were not considered to be due to TRTLE toxicity.

Friedman *et al*. reported that when pregnant and non-pregnant mice of the Swiss Webster strain were fed diets containing 0.1% tomatidine for 14 days (200 mg tomatidine/kg bw/day), the rate of weight gain was significantly reduced and the relative weight of the liver was significantly increased compared with the control diet group (Friedman *et al*., 2003). Prior to the present study, we conducted a preliminary, 14-day repeated oral toxicity study of TRTLE in female mice only, and found no changes in general condition, body weight, food intake, hematology, hematochemistry, or pathological anatomy that suggested an effect of TRTLE. Significant increase in absolute and relative liver weights was observed, but the changes were not accompanied by serum enzyme levels or histopathological changes, that were considered to be of low toxicological significance, although they were attributable to the administration of TRTLE (results not shown). In the 90-day repeated-dose toxicity study in the present study, a significant increase in relative liver weight was observed only in male mice, but no change was observed after the 28-day recovery period, suggesting that the increase or decrease was due to a reversible adaptive response.

As described above, repeated oral administration of 133 mg TRTLE/kg bw/day to mice for 90 days resulted in no toxic effects due to TRTLE. Therefore, the non-toxic dose (NOAEL) under the conditions of this study was determined to be 133 mg TRTLE/kg bw/day (tomatidine equivalent: 80 mg/kg bw/day) for both males and females. No effects related to the administration of TRTLE were observed after the 28-day recovery period, and no delayed toxicity was observed. According to the results of a risk assessment of glycoalkaloids recently reported by the European Food Safety Authority (Schrenk *et al*., 2020), there were no significant effects in rats treated with 20 mg tomatidine/kg bw/day for 200 days (Wilson *et al*., 1961), and two studies with mice have shown that the lowest toxic dose (LOAEL) of tomatidine is 200 mg/kg bw/day (Friedman *et al*., 1996, 2003).

The Ames test confirmed that TRTLE does not have genotoxic effects, with or without a metabolic activation system. In other study performing the Ames test on glycoalkaloids, it has been reported that α-solanine and solanidine from potato edible parts do not have genotoxic effects (Friedman and Henika, 1992). However, there have been no cases in which α-tomatine has been evaluated (Schrenk *et al*., 2020), and the same is true for tomatidine. Therefore, the results of this study are novel in that tomatidine derived from α-tomatine was used as the test substance, and that it was obtained from a leaf for which there is limited experience as a food.

The results obtained thus suggest that TRTLE does not exhibit toxicity under the investigated conditions and suggest that it is a suitable candidate for further investigation regarding its potential use as a functional food ingredient.

ACKNOWLEDGMENTS

This research was conducted as part of a regional revitalization project of the Shizuoka Industrial Promotion Foundation and the Shizuoka Prefecture Advanced Agriculture Project ("AOI Project") and was supported by Shizuoka Prefecture.

The single-dose toxicity study was conducted at the Japan Food Analysis Center (Tama Laboratory, Tokyo, Japan). The 90-day repeated-dose toxicity study was conducted at BioSafety Research Center Inc. (Shizuoka, Japan) (study director: Mai Sugi). The Ames test was conducted at BML Inc. (BML General Laboratory, Saitama, Japan) (study director: Tomoko Sarada) under contract with New Drug Research Center Inc. (Hokkaido, Japan) (study director: Naoaki Takahashi).

Conflict of interest---- Takanori Suzuki, Nobuya Yanai and Shigenobu Shiotani are inventors of the Japan patent JP7085262B2 for production method of tomatidine applied by Tokai Bussan Co., Ltd.

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