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Evaluation of Genotoxicity and 28-day Oral Dose Toxicity on Freeze-dried Powder of *Tenebrio molitor* Larvae (Yellow Mealworm)

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The larval form of *Tenebrio molitor* (*T. molitor*) has been eaten in many countries and provides benefits as a new food source of protein for humans. However, no information exists regarding its safety for humans. The objective of the present study was to evaluate the genotoxicity and repeated dose oral toxicity of the freeze-dried powder of *T. molitor* larvae. The genotoxic potential was evaluated by a standard battery testing: bacterial reverse mutation test, *in vitro* chromosome aberration test, and *in vivo* micronucleus test. To assess the repeated dose toxicity, the powder was administered once daily by oral gavage to Sprague-Dawley (SD) rats at dose levels of 0, 300, 1000 and 3000 mg/kg/day for 28 days. The parameters which were applied to the study were mortality, clinical signs, body and organ weights, food consumption, ophthalmology, urinalysis, hematology, serum chemistry, gross findings and histopathologic examination. The freeze-dried powder of *T. molitor* larvae was not mutagenic or clastogenic based on results of *in vitro* and *in vivo* genotoxicity assays. Furthermore, no treatment-related changes or findings were observed in any parameters in rats after 28 days oral administration. In conclusion, the freeze-dried powder of *T. molitor* larvae was considered to be non-genotoxic and the NOAEL (No Observed Adverse Effect Level) was determined to be 3000 mg/kg/day in both sexes of SD rats under our experimental conditions.

Key words: Edible insect, Genotoxicity, Repeated dose toxicity, *Tenebrio molitor* larvae

INTRODUCTION

The world population has been increasing rapidly, which has developed worldwide issues such as carbon dioxide emissions and food shortages. The increasing demand for animal products has raised energy demands such as coal, gas, and oil, which has led to increasing levels of carbon dioxide in the atmosphere (1). In addition, the food shortage problem is growing increasingly and prevalent throughout the world. Therefore, there have recently been many attempts to increase livestock productivity or food efficiency and find new sources of food (2-4). Insects have been eaten in many countries as a traditional source of protein and food for the promotion of health. Entomophagy, the consumption of insects, has many benefits for health, environmental, and economic factors (5). Over 1000 different species of insect are eaten worldwide, and the insects are rich in protein and good fatty acids (6). Furthermore, insects emit fewer greenhouse gases than most livestock and are very efficient at converting feed into protein. Therefore, many papers have been reported that insects could be a key to solving the world's food problems (7-9). There are many methods to cook and eat insects, but they are usually consumed as a whole body in their larval or adult form. However, food safety aspects and the potential toxicity of the insects has been called into a question (10).

T. molitor, whose larvae are known as yellow mealworms, belong to the order Coleoptera of the family Tenebrionidae. The larvae have been used as a food for carnivore reptile pets, birds and other animals. *T. molitor* is much larger than other insects and can easily be handled (3). In addition, it has the advantage of being easy to rear, having a short life cycle, and the larvae have a high protein content (11,12). Based on the above studies, *T. molitor* larva is a good, novel source of food for humans. The protein components have been established as having future food applica-

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tions in other study (13). However, no information exists regarding the safety or toxicity of *T. molitor* larva for human use. Therefore, the objective of the present study was to evaluate their genotoxicity in bacterial reverse mutation test, *in vitro* chromosome aberration test, *in vivo* micronucleus test, and 28-day oral dose toxicity test in male and female Sprague-Dawley (SD) rats with the freeze-dried powder of *T. molitor* larvae. The present study was performed in compliance with the Good Laboratory Practice (GLP) of the Organization for Economic Cooperation and Development (OECD) and the Korea Food and Drug Administration (KFDA). The protocols for animal studies were reviewed by the Institutional Animal Care and Use Committee (IACUC) of the Korea Institute of Toxicology (KIT), which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

MATERIALS AND METHODS

Preparation of the freeze-dried powder of T. molitor larvae (fdTML) and analysis. The larvae of T. *molitor* were obtained from Sworm Farm (Cheonan, Chungchengnam-do, Korea), freeze-dried and ground to a powder, then sterilized by World Way Co. (Yeongi, Chungchengnam-do, Korea). The fdTML was analyzed as safe with respect to food poisoning pathogen contamination by assessing *Escherichia coli* O158:H7, *Salmonella* spp., and heavy metal (i.e., Pb, Hg, As and Cd) content. The food poisoning pathogens, Pb, Hg, and Cd levels were not detected with fdTML and the As level was 0.03 mg/kg, which was lower than the standard index for food (13).

The general components were measured using the official methods of analysis of the Association of Official Analytical Chemists, and the marker compounds were measured using gas chromatography. Oleic acid, the marker compound of *T. molitor*, was 51.40 ± 0.47% (13).

Formulation of fdTML. The appropriate amount of fdTML for the highest dose was weighed and added into the tubes containing sterile distilled water (Daihan Pharm., Republic of Korea) for bacterial reverse mutation testing and *in vitro* chromosome aberration test, and mixed with a vortex mixer for at least 10 min. For oral administration in mice or rats, the appropriate amount of fdTML powder was measured and suspended in distilled water for the highest dose group, and this suspension was further diluted to prepare a lower dose suspension.

Bacterial reverse mutation test. A mutagenic potential of fdTML was examined in the absence and presence of S-9 mixture using histidine-requiring *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537 strains, and tryptophan-requiring *Escherichia coli* WP2uvrA strain. All strains were purchased from Molecular Toxicology Inc. (USA). The viable cell counts in the bacterial cultures used in the test were more than 1×10^9 cells per milliliter. The dose range-finding (DRF) test was carried out at five concentration levels of 8, 40, 200, 1000, 3000, and 5000 μg/plate, including vehicle and strain-specific positive controls in the absence and presence of S-9 mixture. No antibacterial effects (cytotoxicity), defined as the diminution of background lawn, formation of microcolonies, or the ≥ 50% reduction in the mean number of revertants per plate relative to the mean vehicle control values were observed. However, the precipitation of test article was observed at ≥ 1000 μg/plate concentrations in all strains when the formulations were mixed with top agar and incubated for 48 hrs.

Based on these results, 5000 μg/plate was selected as the highest concentration for the confirmatory test, considering the test article was dietary supplements, and two-fold serial dilutions were performed to yield six concentration levels. The number of revertant colonies was counted by the unaided eye. The results were expressed as the mean number of revertant colonies from the triplicate plates per concentration with the standard deviation and ratio of the mean vehicle control value.

In vitro chromosome aberration test in Chinese hamster lung (CHL) cells. Chinese hamster lung (CHL/IU) cell line was purchased from the American Type Culture Collection (ATCC, USA). That cell line was chosen because it has been the most frequently used for *in vitro* chromosome aberration testing for regulatory submissions due to its high detection sensitivity.

The DRF test was performed with nine concentration levels of 19.5, 39.1, 78.1, 156.3, 312.5, 625, 1250, 2500 and 5000 μg/ml, including the vehicle control in the presence of the S-9 mixture (6 hrs treatment), and in the absence of S-9 mixture (6 and 22 hrs treatment) in order to determine the relative toxicity of fdTML to the cell cultures. From the results of the DRF test, the precipitation or turbidity/precipitation of test article was observed at all concentrations at both the beginning and end of the treatment. There was also cytotoxicity at the concentration ranges at which the severe turbidity and precipitation of the test articles were shown. In the results of pH and osmolality measurement for the culture medium at the beginning and the end of treatment, a pH change of more than one unit or a change in osmolality of more than 50 mOsm/kg (as compared to the vehicle) was not observed at the highest concentration (5000 μg/ml) of each treatment group.

Based on these results, the concentration range for the confirmatory test was designed to consider the precipitation of fdTML. The treatment at each concentration was conducted in duplicate. The slides were prepared following the hypotonic-methanol-glacial acetic acid-flame drying-giemsa stain schedule for metaphase plate analysis. At least 200 well-spread intact metaphases were scored per concentra-

tion under 1000× magnification, using a biological microscope of differential interference types.

The identification of chromosomal aberrations was conducted as follows, the types of chromosome aberration were classified into two groups: Structural aberration and numerical aberration. Structural aberration was subdivided into chromatid break (ctb), chromatid exchange (cte), chromosome break (csb), chromosome exchange (cse), while numerical aberration was subdivided into polyploidy (PP, ≥ 37 chromosomes) and endoreduplication (ER). Two types of aberration such as chromatid and chromosome gap were recorded but not included in the calculation of the aberration rates.

In vivo micronucleus tests in mice. Approximately six-week-old specific pathogen-free mice (CrljOri:CD1 ICR, Male 23.3~29.1 g; Female 22.4~26.8 g) were obtained from Orient Bio Inc. (Seongnam, Gyeonggi-do, Korea) and used after seven days of acclimation. The animals were housed in polycarbonate cages with bedding. The animal room was maintained at a temperature of 22 ± 3°C, a relative humidity of approximately 30~70%, air ventilation of 10~20 times/hr and light intensity of 150~300 lux with 12-hr light-dark cycles. A commercial pellet diet (PMI nutrition International, USA) and sterilized tap water were provided *ad libitum*. The DRF test was performed at four dose levels: 250, 500, 1000, and 2000 mg/kg, which is the limit dose for treatment up to 14 days, according to the OECD guidelines. The treated mice did not show any clinical signs or behavioral alterations during the observation period up to the 2000 mg/kg dosage.

Based on these results, *in vivo* micronucleus tests were conducted at three dose levels (500, 1000, and 2000 mg/20 ml/kg). The fdTML was orally administered twice at 24-hr intervals in both male and female mice (six animals/sex/group) while the positive controls were administered once intraperitoneally at 70 mg/10 ml/kg. All animals were sacrificed by CO_2 gas inhalation at approximately 24 hrs after the final administration. Micronucleus testing was carried out according to the method of Schmid (14). Smears were allowed to dry, fixed with methanol, and stained with a May-Grunwald/Giemsa solution. The stained slides were washed and allowed to air dry. Slides were then examined blind under 1000× magnification. Two thousand PCEs were scored per animal by the same observer to determine the frequencies of micronucleated polychromatic erythrocytes (MNPCEs). PCEs/(PCEs+NCEs) ratio, indicators of cytotoxicity, which was calculated by counting 500 total erythrocytes per animal.

28-day repeated dose oral toxicity study. Twenty-four male- and female-specific pathogen-free SD rats were obtained from Orient Bio Co. at five weeks of age. The animals were acclimatized for 7 days, and healthy animals were selected for the study. Twenty male and female rats were randomly assigned to four groups (one control group and three treatment groups). Each group consisted of five rats of each gender. The body weight range prior to the start of dosing was 205.0~223.7 g for the males, and 144.9~164.5 g for the females. The animals were housed in stainless steel cages throughout the study period. The animal room was maintained in the same condition as in the *in vivo* micronucleus test in mice.

A previous single dose toxicity study performed by the Korea Institute of Toxicology indicated that an fdTML dose of 3000 mg/kg/day was well-tolerated (results not published). Therefore, doses of 0, 300, 1000, and 3000 mg/kg/day were selected for this 28-day repeated dose study. The condition and behavior of all animals was checked once daily throughout the acclimation period. All animals were examined and clinical signs were recorded twice daily both before and after dosing during the treatment period, and once on the day of necropsy.

The animals were weighed prior to the randomization on the day of arrival, before dosing on the first day of dosing and once per week thereafter. Final body weight was measured on the day of necropsy. Cage food consumption was recorded once during the acclimation period and once weekly during the treatment period. Individual food consumption was calculated as g/rat/day. External eye examinations were performed on all animals on week four before necropsy.

Urine samples were collected overnight for 16 hr from animals housed in metabolism cages during the last week of treatment. Each animal was housed in an individual metabolism cage; food was withdrawn overnight, but water was available. Urinalysis was performed using a urine automatic analyzer (Cobas U411 urine analyzer, Cobas, Germany), and urine stick (Multistix 10 SG, Siemens, Germany) to evaluate the following parameters: Urine volume, color, specific gravity, pH, and protein, ketone body, erythrocyte, glucose, bilirubin, nitrite, and urobilinogen content. Microscopic examination of urine cast, epithelial cell, red blood cells, and white blood cell content was also performed.

All animals were fasted overnight prior to the necropsy and blood collection. Blood samples were collected for hematology, coagulation, and serum chemistry from the vena cava of all animals under the isoflurane anesthesia at necropsy. Blood samples were collected into tubes containing EDTA-2K for hematology analysis. White blood cell count, red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentration, platelets, differential leukocyte count (neutrophil, lymphocyte, monocyte, eosinophil, basophil, and large unstained cell), and reticulocyte count were analyzed using the ADVIA 2120i hematology system (Siemens, USA). In addition, blood samples treated with 3.2% sodium citrate were analyzed for prothrombin and activated partial throm-

boplastin time using the ACL 9000 (Instrumental Laboratory, Italy).

Blood samples were collected for serum chemistry analysis in tubes lacking anticoagulant and placed at room temperature for at least 90 min prior to centrifugation (1600 ×g, 10 min). Blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatinine (CREA), glucose (GLU), total cholesterol (TCHO), albumin/globulin ratio (A/G), total protein (TP), albumin (ALB), creatine kinase (CK), triglycerides (TG), total bilirubin (TBIL), phospholipids (PL), and blood levels were measured using an automatic TBA 200FR NEO analyzer (Toshiba Co., Japan).

Following blood sampling, the animals were killed by exsanguination from the vena cava and aorta under isoflurane anesthesia. Complete necropsy examinations were performed on all animals. Absolute organ weights of the brain, pituitary gland, adrenal gland, liver, spleen, kidneys, heart, thymus, lungs, salivary gland, thyroid gland, testes, epididymides, seminal vesicle, prostate, uterus and ovaries were calculated, along with the relative organ weights as a percentage of the terminal body weight.

Following a detailed external and internal examination, all tissues (i.e., testes, epididymides, ovaries, uterus, spleen, kidneys, adrenal gland, liver, thyroid gland, thymus, heart) were taken from each animal. With the exception of the testes and epididymides, all tissues were preserved in a 10% neutral buffered formalin solution. The testes and epididymides were fixed in Bouin's fixative for approximately 48 hr before being transferred to 70% alcohol. The tissues were sectioned and stained with hematoxylin and eosin (H&E) prior to microscopic examination. All preserved tissues from the animals in the vehicle control and the highest dose group were examined.

Statistical analysis. The statistical analyses for *in vitro* chromosomal aberration results were done using Statistical Analysis System software (version 9.2, SAS Institute Inc., USA). The number of aberrant metaphases (excluding gaps) and the number of [PP+ER] were analyzed. Statistical evaluation of the *in vivo* micronucleus results was accepted

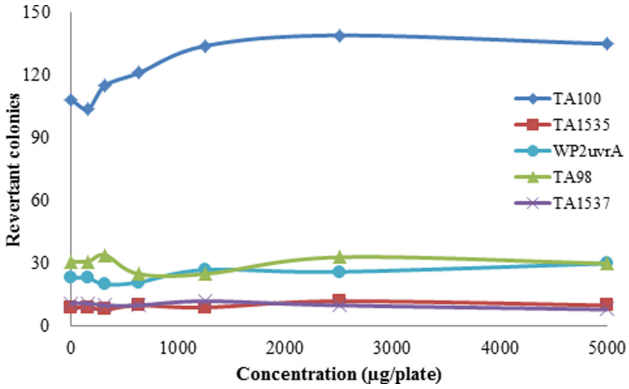

Fig. 1. Dose response curve of revertant colonies by fdTML in the presence of S-9 mixture. Five strains (*S. typhimurium* TA98, TA100, TA1535, TA1537, and *E. coli* WP2uvrA) were exposed to fdTML and incubated for 48 hrs. Data were expressed as the mean values of colonies from triplicate plates per concentration.

Table 1. Results of bacterial reverse mutation testing for fdTML in the absence of S-9 mixture

Concentration (μg/plate)	Revertant colonies per plate (Plate A, B and C) (Mean ± SD) [Factor][a]				
	Base substitution type			Frameshift type	
	TA100	TA1535	WP2*uvr*A	TA98	TA1537
0	97, 101, 108 102 ± 6	9, 9, 10 9 ± 1	18, 20, 21 20 ± 2	15, 20, 23 19 ± 4	4, 7, 7 6 ± 2
156.3	103, 111, 121 112 ± 9 [1.1]	9, 10, 12 10 ± 2 [1.1]	19, 22, 27 23 ± 4 [1.2]	14, 22, 22 19 ± 5 [1.0]	6, 6, 6 6 ± 0 [1.0]
312.5 #	105, 110, 117 111 ± 6 [1.1]	11, 12, 12 12 ± 1 [1.3]	19, 20, 24 21 ± 3 [1.1]	15, 15, 21 17 ± 3 [0.9]	5, 7, 7 6 ± 1 [1.0]
625 #	110, 112, 121 114 ± 6 [1.1]	8, 8, 12 9 ± 2 [1.0]	19, 19, 23 20 ± 2 [1.0]	13, 19, 21 18 ± 4 [0.9]	5, 6, 7 6 ± 1 [1.0]
1250 #	105, 118, 124 116 ± 10 [1.1]	11, 12, 13 12 ± 1 [1.3]	23, 25, 29 26 ± 3 [1.3]	14, 19, 21 18 ± 4 [0.9]	8, 8, 8 8 ± 0 [1.3]
2500 #	135, 137, 145 139 ± 5 [1.4]	9, 10, 16 12 ± 4 [1.3]	20, 25, 30 25 ± 5 [1.3]	15, 17, 19 17 ± 2 [0.9]	5, 6, 7 6 ± 1 [1.0]
5000 #	144, 148, 160 151 ± 8 [1.5]	8, 10, 11 10 ± 2 [1.1]	23, 24, 30 26 ± 4 [1.3]	15, 15, 23 18 ± 5 [0.9]	4, 7, 7 6 ± 2 [1.0]
Positive control	453, 461, 475 463 ± 11 [4.5]	205, 212, 224 214 ± 10 [23.8]	481, 509, 510 500 ± 16 [25.0]	282, 301, 317 300 ± 18 [15.8]	170, 181, 196 182 ± 13 [30.3]

[a]Number of revertant colonies of the treated plates/Number of revertant colonies of the vehicle control plate.

when all of the PCEs/(PCEs+NCEs) ratios were greater than 0.1. The results were judged positive when there was a statistically significant dose-related increase, or a reproducible increase in the frequencies of MNPCEs (in *in vivo* micronucleus test) or aberrant metaphases (in *in vitro* chromosome aberration test) at least at one dose level. The results of the statistical evaluation were regarded as significant when the P value was less than 0.05. No statistical analyses were performed on bacterial reverse mutation test results.

The animal data collected during the study were analyzed for variance homogeneity using Bartlett's test. Homogeneous data were analyzed using the Analysis of Variance (ANOVA) test, and the significance of inter-group differences were analyzed using Dunnett's t-test. Heterogeneous data were analyzed using the Kruskal-Wallis test, and the significance of the inter-group differences between the control and treated groups were assessed using Dunn's rank sum test. Statistical analyses were performed by comparing the dose groups to the vehicle control group using the Path/Tox system. The results of the comparisons are only indicated when p-values of less than 0.05 or 0.01 were attained.

Table 2. Results of *in vitro* chromosome aberration test for fdTML

S9 mix time[a]	Nominal concentration (μg/ml)	No. of findings/100 metaphases							No. of aberrant metaphases	Mean aberrant metaphases
		ctb	csb	cte	cse	other	PP	ER		
S9 mix (-) 6+18 hrs	0[b]	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	
	5 #	1	0	0	0	0	0	0	1	1.5
		0	1	1	0	0	0	0	2	
	10 #	0	0	0	0	0	0	0	0	0.5
		0	0	1	0	0	1	0	1	
	20 #	0	0	0	0	0	0	0	0	0.0
		0	0	0	0	0	1	0	0	
	CPA 6	7	1	32	0	1	0	0	29	32.5
		9	0	42	1	3	1	0	36	
S9 mix (+) 6+18 hrs	0	1	0	0	0	1	0	0	2	1.5
		0	0	0	0	1	0	0	1	
	5 #	0	2	0	0	1	1	0	2	1.0
		0	0	0	0	0	1	0	0	
	10 #	1	2	0	0	0	2	0	2	1.5
		0	1	0	0	0	2	0	1	
	20 #	0	1	0	0	0	2	0	1	2.0
		0	4	0	0	0	0	0	3	
	EMS 800	5	1	37	0	1	1	0	29	27.5
		3	1	30	0	4	0	0	26	
S9 mix (-) 22+0 hrs	0	0	1	0	0	0	1	0	1	1.0
		0	1	0	0	0	2	0	1	
	5 #	0	0	0	0	0	0	0	0	0.5
		0	1	0	0	0	1	0	1	
	10 #	1	2	0	0	0	1	0	2	1.0
		0	0	0	0	0	1	0	0	
	20 #	0	0	0	0	0	2	0	0	1.0
		1	0	0	1	0	1	0	2	
	EMS 600	13	0	68	0	5	0	0	43	44.5
		17	3	55	0	3	1	0	46	

[a]Treatment time+Recovery time.
[b]Vehicle: Water, sterile-filtered, BioReagent, suitable for cell culture.
#: Precipitation at the beginning and the end of treatment.
CPA: Cyclophosphamide monohydrate, EMS: Ethyl methanesulfonate, ctb: chromatid break, csb: chromosome break, cte: chromatid exchange, cse: chromosome exchange, PP: polyploid, ER: endoreduplication.
other (multiple aberrations) indicate metaphases with more than four same-type aberrations.

RESULTS AND DISCUSSION

The results of the bacterial reverse mutation test of fdTML are shown in Fig. 1 and Table 1. No contaminant colonies were observed on the sterility plates for the highest concentrations of fdTML formulation and S-9 mixture. The number of revertant colonies in all strains was not increased more than twice (for strains TA98, TA100, and WP2uvrA) or three times (for strains TA1535 and TA1537) compared to the vehicle control in the absence or presence of S-9 mixture. However, the precipitation of test articles was observed at 312.5 μg/plate concentrations in the absence or presence of S-9 mixture when the formulations were mixed with top agar and incubated for 48 hrs. The positive controls induced significant increases (defined as four times that of the vehicle control) in the number of revertant colonies, which indicates that the test was valid.

The number of metaphases with structural and numerical aberrations is presented in Table 2. The number of aberrant metaphases at the concentration range of fdTML-tested was 0.0%~2.0% for the short-term (6 hrs) treatment in the absence or presence of S-9 mixture, and 0.5%~1.0% for the continuous (22 hrs) treatment in the absence of S-9 mixture. Therefore, the number of aberrant metaphases was not increased at any concentration, regardless of the presence of S-9 mixture compared with that of the vehicle control. The number of aberrant metaphases in positive control was 32.5%, 27.5%, and 44.5% for short-term treatment in the absence or presence of S-9 mixture and the continuous treatment in the absence of S-9 mixture, respectively, indicating that the test was valid.

Table 3 showed the frequencies of the micronucleated polychromatic erythrocytes per 2000 polychromatic erythrocytes (PCEs) and the ratios of PCEs per 500 erythrocytes (PCEs/[PCEs+NCEs]) as an indicator of cytotoxicity. The frequencies of MNPCEs were 1.50, 0.67, 1.67, and 0.50 in the order of vehicle control, 500, 1000, and 2000 mg/kg for the male mice, respectively, and were 1.00, 1.17, 1.17 and 1.00 in the order of vehicle control, 500, 1000, and 2000 mg/kg for the female mice, respectively. Therefore, no statistically significant increase in the frequencies of micronucleated polychromatic erythrocytes was observed for any treated group compared to the vehicle control group. The proportions of PCEs/(PCEs+NCEs) were 0.54, 0.61, 0.55, and 0.62 in the order described above in male mice, and were 0.64, 0.65, 0.59, and 0.60 in the order described above in the female mice, showing no significant difference between

Table 3. The effects of fdTML on the formation of micronucleated polychromatic erythrocytes in the bone marrow of male and female mice

Dose (mg/kg)	Number of animal	MNPCEs/2000 PCEs (Mean ± S.D.)	PCEs/(PCEs+NCEs) (Mean ± S.D.)
Male			
0	6	1.50 ± 1.05	0.54 ± 0.07
500	6	1.67 ± 1.03	0.61 ± 0.12
1000	6	1.67 ± 1.03	0.55 ± 0.10
2000	6	0.50 ± 0.84	0.62 ± 0.07
CPA 70	6	96.83 ± 16.09	0.50 ± 0.04
Female			
0	6	1.00 ± 0.89	0.64 ± 0.06
500	6	1.17 ± 0.98	0.65 ± 0.08
1000	6	1.17 ± 0.75	0.59 ± 0.06
2000	6	1.00 ± 0.89	0.60 ± 0.09
CPA 70	6	64.00 ± 13.37	0.48 ± 0.07

PCEs: Polychromatic erythrocytes, NCEs: Normochromatic erythrocytes, MNPCEs: PCEs with one or more micronuclei, CPA: Cyclophosphamide monohydrate.

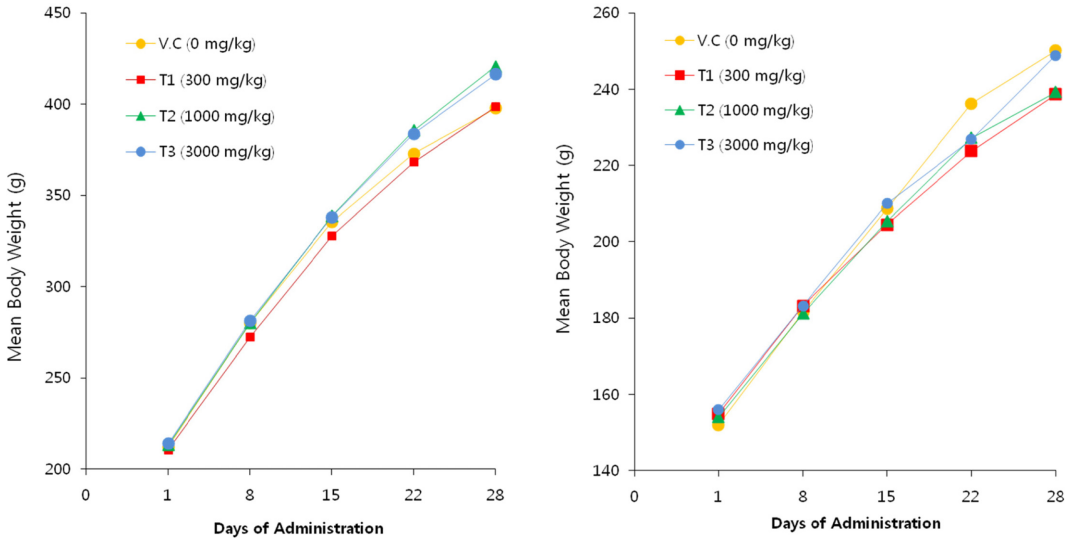

Fig. 2. Body weight changes of males (left) and females (right) treated with fdTML for 28 days.

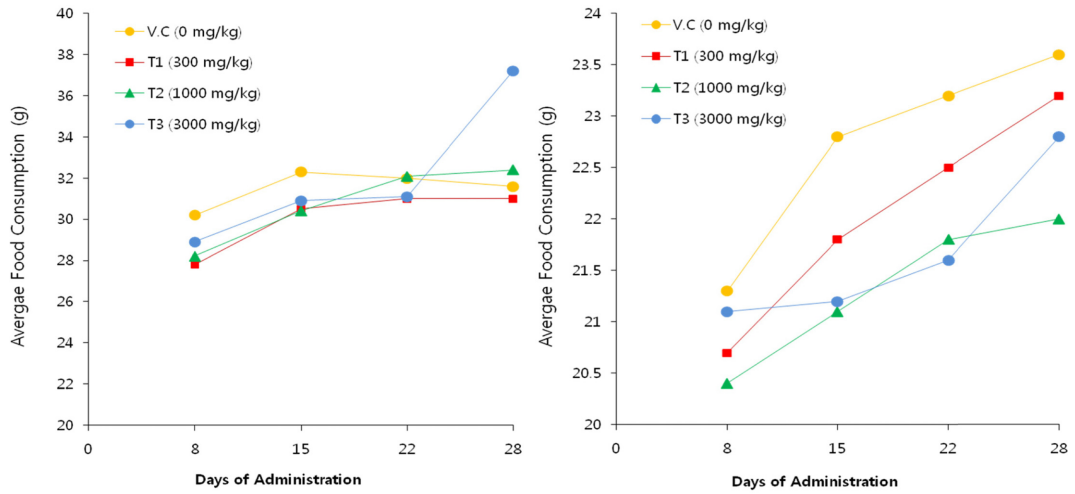

Fig. 3. Food consumption data of males (left) and females (right) treated with fdTML for 28 days.

Table 4. Hematological values of rats treated orally with fdTML for 28 days

Parameters	Vehicle control	300 mg/kg	1000 mg/kg	3000 mg/kg
Males				
WBC ($\times 10^3/\mu l$)	7.83 ± 1.733	8.40 ± 1.242	9.35 ± 1.427	8.73 ± 2.644
RBC ($\times 10^6/\mu l$)	8.22 ± 0.264	7.92 ± 0.345	7.99 ± 0.166	8.32 ± 0.261
HGB (g/dl)	15.6 ± 1.37	15.7 ± 0.23	15.8 ± 0.47	15.8 ± 0.30
HCT (%)	50.2 ± 1.52	48.8 ± 1.88	47.8 ± 1.08	48.6 ± 1.87
MCV (fl)	61.1 ± 2.30	61.7 ± 1.48	59.9 ± 2.34	58.3 ± 1.05
MCH (pg)	19.0 ± 1.21	19.9 ± 0.72	19.8 ± 0.56	18.9 ± 0.35
MCHC (g/dl)	31.1 ± 2.45	32.3 ± 0.85	33.1 ± 1.03	32.5 ± 0.69
PLT ($\times 10^3/\mu l$)	1302. ± 242.0	1263. ± 65.9	1395. ± 77.4	1388. ± 111.2
RET% (%)	2.70 ± 0.455	3.01 ± 0.143	3.57 ± 1.377	2.80 ± 0.326
NEU% (%)	11.5 ± 4.17	15.6 ± 8.24	13.0 ± 4.16	10.8 ± 2.06
LYM% (%)	84.3 ± 5.14	80.4 ± 8.17	82.5 ± 4.73	85.0 ± 2.39
EOS% (%)	0.6 ± 0.22	0.7 ± 0.24	0.7 ± 0.26	0.7 ± 0.23
MON% (%)	2.2 ± 0.91	1.9 ± 0.46	2.6 ± 0.86	2.1 ± 0.37
BAS% (%)	0.5 ± 0.12	0.4 ± 0.11	0.4 ± 0.13	0.4 ± 0.09
LUC% (%)	0.9 ± 0.24	1.0 ± 0.19	0.8 ± 0.15	1.0 ± 0.23
PT (sec)	15.8 ± 0.75	15.7 ± 0.45	16.1 ± 0.43	16.7 ± 0.82
APTT (sec)	16.4 ± 0.43	15.7 ± 0.92	16.1 ± 0.71	16.1 ± 1.36
Females				
WBC ($\times 10^3/\mu l$)	8.56 ± 2.390	6.84 ± 0.959	7.11 ± 0.686	8.12 ± 1.904
RBC ($\times 10^6/\mu l$)	8.06 ± 0.362	8.14 ± 0.320	7.77 ± 0.300	7.74 ± 0.362
HGB (g/dl)	15.9 ± 0.78	16.1 ± 0.43	15.3 ± 0.55	15.3 ± 0.48
HCT (%)	47.4 ± 2.06	48.0 ± 0.86	46.2 ± 1.28	46.0 ± 1.11
MCV (fl)	58.8 ± 0.53	59.0 ± 2.63	59.4 ± 1.09	59.4 ± 1.61
MCH (pg)	19.7 ± 0.26	19.7 ± 1.13	19.7 ± 0.25	19.8 ± 0.72
MCHC (g/dl)	33.5 ± 0.38	33.4 ± 0.45	33.2 ± 0.31	33.3 ± 0.47
PLT ($\times 10^3/\mu l$)	1253. ± 113.6	1273. ± 202.7	1277. ± 121.2	1329. ± 162.8
RET% (%)	2.58 ± 0.559	2.61 ± 0.368	2.44 ± 0.645	2.80 ± 0.369
NEU% (%)	12.2 ± 6.20	14.4 ± 7.08	10.0 ± 3.61	13.4 ± 7.88
LYM% (%)	84.0 ± 6.53	82.1 ± 6.79	86.3 ± 3.70	82.5 ± 7.54
EOS% (%)	0.8 ± 0.32	1.0 ± 0.33	0.7 ± 0.28	0.7 ± 0.16
MON% (%)	1.6 ± 0.64	1.3 ± 0.24	1.6 ± 0.27	1.6 ± 0.17
BAS% (%)	0.4 ± 0.11	0.4 ± 0.08	0.4 ± 0.14	0.5 ± 0.19
LUC% (%)	0.9 ± 0.13	0.8 ± 0.24	1.0 ± 0.29	1.3 ± 0.33
PT (sec)	16.9 ± 0.31	16.1 ± 1.83	15.4 ± 0.26	15.5 ± 0.64
APTT (sec)	13.5 ± 1.39	13.8 ± 1.28	13.2 ± 1.57	13.7 ± 1.19

Each value represents the mean ± SD for five rats.

the vehicle control and treated groups. The frequencies of micronucleated polychromatic erythrocytes in positive control group were 96.83 and 64.00 in male and female mice, respectively. The frequencies of micronucleated polychromatic erythrocytes were significantly increased in contrast with that of the vehicle control group, indicating that the present study was performed under acceptable experimental conditions and is valid.

Edible insects are regarded as a promising source of protein and other nutrients for humans. The nutrients and the prospective aspects as a source of food have already been established through many study results. However, there has been no information regarding the toxicity or safety of edible insects as they are. To assess the toxicity of fdTML, SD rats were orally treated with 300, 1000, or 3000 mg/kg/day of fdTML, or a vehicle. Mortality, clinical signs, body weight, food consumption, ophthalmologic examination, hematology, serum chemistry, urinalysis, gross findings, organ weights, and microscopic findings were observed. In addition, as a series of safety studies, a battery of three classical genotoxicity tests were carried out in this study, because no single test can detect every genotoxic agent (EMEA/HMPC/107079, 2007).

In 28-day repeated oral toxicity study, there were no treatment-related mortalities or clinical signs in any groups throughout the study period. In addition, there were no treatment-related changes in body weight for either gender of rat (Fig. 2). In males, the body weight showed a tendency to increase in the mid (1000 mg/kg/day) and high dose (3000 mg/kg/day) groups up to 106%, compared to that of the vehicle control. It seems to be related to the food consumption data in mid- and high-dose males (Fig. 3). However, the food consumption data showed no clear differences between the vehicle control and the treated groups, except for the high-dose males. The increases in body weight and food consumption in mid- and high-dose male groups were different from those of the female groups. In other toxicity studies using oleic acid, there were no treatment-related changes in body weight or food consumption for any of the groups (15). Therefore, the changes in body weight and food consumption are not considered related to the treatment, and will necessitate further study of the long-term results. In ophthalmologic examination, no abnormal findings were observed for any of the groups.

Table 5. Serum chemistry values of rats treated orally with fdTML for 28 days

Parameters	Vehicle control	300 mg/kg	100 mg/kg	3000 mg/kg
Males				
GLU (mg/dl)	107.4 ± 21.31	105.7 ± 16.50	110.5 ± 15.42	122.2 ± 27.54
BUN (mg/dl)	12.6 ± 1.03	13.8 ± 1.61	14.4 ± 0.98	12.6 ± 1.65
CREA (mg/dl)	0.53 ± 0.031	0.57 ± 0.021	0.55 ± 0.039	0.55 ± 0.029
TP (g/dl)	6.62 ± 0.301	6.39 ± 0.209	6.69 ± 0.218	6.38 ± 0.335
ALB (g/dl)	4.17 ± 0.090	4.05 ± 0.078	4.17 ± 0.186	4.03 ± 0.162
A/G (ratio)	1.71 ± 0.170	1.74 ± 0.112	1.66 ± 0.120	1.71 ± 0.093
AST (IU/L)	120.6 ± 7.38	120.3 ± 11.27	121.9 ± 7.63	127.5 ± 16.64
ALT (IU/L)	32.2 ± 2.22	32.6 ± 2.20	31.3 ± 2.37	33.1 ± 4.04
TBIL (mg/dl)	0.130 ± 0.0122	0.122 ± 0.0070	0.144 ± 0.0276	0.107 ± 0.0126
ALP (IU/L)	606.4 ± 79.51	535.6 ± 74.23	430.6 ± 104.84	580.0 ± 121.17
CK (IU/L)	648. ± 135.7	669. ± 178.2	825. ± 64.2	743. ± 196.9
TCHO (mg/dl)	53. ± 6.8	60. ± 16.7	69. ± 6.6	60. ± 14.2
TG (mg/dl)	38.2 ± 17.57	47.5 ± 15.63	61.0 ± 13.27	49.6 ± 13.79
PL (mg/dl)	84. ± 11.6	95. ± 18.2	105. ± 9.2	92. ± 16.6
Females				
GLU (mg/dl)	89.9 ± 38.09	104.4 ± 27.92	111.0 ± 36.67	95.1 ± 34.19
BUN (mg/dl)	15.6 ± 1.60	16.1 ± 1.95	14.4 ± 1.94	18.1 ± 3.32
CREA (mg/dl)	0.54 ± 0.046	0.60 ± 0.073	0.57 ± 0.049	0.65 ± 0.088
TP (g/dl)	6.63 ± 0.196	6.80 ± 0.338	6.77 ± 0.279	6.55 ± 0.378
ALB (g/dl)	4.29 ± 0.116	4.35 ± 0.155	4.31 ± 0.177	4.22 ± 0.218
A/G (ratio)	1.82 ± 0.065	1.77 ± 0.128	1.75 ± 0.110	1.81 ± 0.076
AST (IU/L)	124.0 ± 26.77	125.4 ± 18.70	130.0 ± 34.87	123.8 ± 19.40
ALT (IU/L)	25.3 ± 2.09	27.7 ± 4.38	28.4 ± 4.42	25.0 ± 2.99
TBIL (mg/dl)	0.154 ± 0.0122	0.135 ± 0.0049	0.139 ± 0.0237	0.118 ± 0.0175
ALP (IU/L)	326.8 ± 65.65	364.4 ± 66.52	354.8 ± 95.26	370.1 ± 74.51
CK (IU/L)	596. ± 158.2	738. ± 265.0	750. ± 336.4	624. ± 184.4
TCHO (mg/dl)	76. ± 19.9	72. ± 21.8	79. ± 7.1	63. ± 11.7
TG (mg/dl)	25.9 ± 4.85	31.6 ± 8.94	30.5 ± 5.78	27.5 ± 3.32
PL (mg/dl)	127. ± 21.7	121. ± 28.2	127. ± 10.3	109. ± 16.4

Each value represents the mean ± SD for five rats.

Table 6. Organ weight values of rats treated orally with fdTML for 28 days

Parameters	Vehicle control	300 mg/kg	1000 mg/kg	3000 mg/kg
Males				
Adrenal gland (g)	0.066 ± 0.0072	0.063 ± 0.0156	0.064 ± 0.0079	0.069 ± 0.0131
Brain (g)	1.979 ± 0.1256	1.999 ± 0.0853	1.982 ± 0.0335	2.006 ± 0.1253
Heart (g)	1.284 ± 0.0888	1.255 ± 0.0629	1.270 ± 0.0864	1.337 ± 0.1459
Kidneys (g)	2.974 ± 0.2927	3.237 ± 0.2883	3.296 ± 0.1284	3.255 ± 0.2160
Liver (g)	12.450 ± 1.9652	12.214 ± 0.6688	12.743 ± 0.9599	12.705 ± 0.2375
Pituitary gland (g)	0.010 ± 0.0013	0.012 ± 0.0024	0.012 ± 0.0011	0.013 ± 0.0030
Prostate (g)	0.472 ± 0.1187	0.492 ± 0.1013	0.530 ± 0.1065	0.565 ± 0.1500
Spleen (g)	0.736 ± 0.0512	0.713 ± 0.0702	0.747 ± 0.1530	0.685 ± 0.0552
Testes (g)	3.393 ± 0.5291	3.216 ± 0.1743	3.268 ± 0.1524	3.361 ± 0.2663
Thymu s(g)	0.584 ± 0.1113	0.543 ± 0.0754	0.586 ± 0.1176	0.583 ± 0.1207
Epididymides (g)	1.023 ± 0.0773	1.031 ± 0.0884	1.020 ± 0.0359	1.063 ± 0.0767
Lung (g)	1.418 ± 0.1492	1.383 ± 0.0913	1.454 ± 0.0992	1.469 ± 0.0824
Seminal vesicle (g)	1.514 ± 0.3535	1.351 ± 0.2055	1.218 ± 0.1893	1.695 ± 0.2673
Thyroid/parathyroid(g)	0.025 ± 0.0081	0.023 ± 0.0060	0.021 ± 0.0043	0.028 ± 0.0065
Salivary gland (g)	0.674 ± 0.0643	0.637 ± 0.0588	0.681 ± 0.0207	0.699 ± 0.0794
Females				
Adrenal gland (g)	0.081 ± 0.0101	0.073 ± 0.0091	0.070 ± 0.0057	0.076 ± 0.0074
Brain (g)	1.881 ± 0.0702	1.833 ± 0.0429	1.786 ± 0.0341	1.849 ± 0.0076
Heart (g)	0.835 ± 0.0651	0.819 ± 0.0954	0.849 ± 0.0883	0.856 ± 0.0702
Kidneys (g)	2.179 ± 0.1332	2.013 ± 0.2710	2.006 ± 0.3702	2.128 ± 0.1407
Liver (g)	7.734 ± 0.6006	7.477 ± 0.9403	7.576 ± 0.9666	7.887 ± 0.9048
Ovaries (g)	0.104 ± 0.0155	0.099 ± 0.0121	0.084 ± 0.0072	0.108 ± 0.0220
Pituitary gland (g)	0.014 ± 0.0022	0.013 ± 0.0030	0.012 ± 0.0024	0.013 ± 0.0027
Spleen (g)	0.483 ± 0.0301	0.479 ± 0.0851	0.447 ± 0.0926	0.529 ± 0.1217
Thymus (g)	0.573 ± 0.1104	0.460 ± 0.0719	0.537 ± 0.1970	0.522 ± 0.1683
Lung (g)	1.165 ± 0.0659	1.104 ± 0.1255	1.186 ± 0.1075	1.164 ± 0.0952
Thyroid/parathyroid (g)	0.024 ± 0.0030	0.019 ± 0.0043	0.021 ± 0.0033	0.020 ± 0.0016
Uterus/cervix (g)	0.508 ± 0.1461	0.588 ± 0.1434	0.701 ± 0.3692	0.616 ± 0.1721
Salivary gland (g)	0.488 ± 0.0494	0.431 ± 0.0390	0.461 ± 0.0641	0.447 ± 0.0288

Each value represents the mean ± SD for five rats.

No significant changes in urinalysis parameters were observed for any treated groups in the study (data not shown). In addition, there were no significant changes in hematology (Table 4) or serum chemistry (Table 5) in any of the treated groups.

Absolute organ weights are shown in Table 6. There were no treatment-related changes in either the absolute or relative organ weights for any of the treated animals. In addition, no treatment-related macroscopic or microscopic findings were observed in any of the treated animals. A minimal dilation of the kidney pelvis was observed in one animal in the vehicle control group. It was considered a spontaneous finding, because it was observed in only one animal in the vehicle control group.

In conclusion, the fdTML did not cause any mutagenic response in four *Salmonella typhimurium* and one *Escherichia coli* strain. There were no clastogenicity in *in vitro* chromosome aberration tests using Chinese hamster lung cells, or in micronucleus test for mice. Our results suggest that fdTML is safe for normal use in terms of genotoxicity. In addition, oral administration of fdTML for four weeks revealed no adverse effects in SD rats up to 3000 mg/kg/day and therefore, the NOAEL (No Observed Adverse Effect Level) was determined to be 3000 mg/kg/day for both sexes of rats in our experimental conditions.

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