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Toxicological safety evaluation of freeze-dried *Protaetia brevitarsis* larva powder

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ABSTRACT

White-spotted flower chafer (*Protaetia brevitarsis*) is an edible insect and its larva was used as a traditional Asian medicine. It's a promising material as a novel food source because of its nutritional components. In this study, as part of the preclinical toxicity program, we evaluated the toxicity of freeze-dried *P. brevitarsis* larva powder to develop a novel food material. In a single-dose oral toxicity study in rats, there were no changes in mortality, clinical observations, and body weight in rats administered 5000 mg/kg *P. brevitarsis* larva powder. In a 13-week oral repeated dose toxicity study in rats, there were no adverse effects or changes in mortality, clinical observations, body weight, food consumption, ophthalmology, clinical pathology, necropsy, organ weight, and histopathology at doses of 300, 1000, and 3000 mg/kg/day. In identification of allergic reactions, *P. brevitarsis* larva powder induced no increases of serum immunoglobulin E and histamine concentrations over 13 weeks of oral administration in rats. In a genotoxicity assessment, *P. brevitarsis* larva powder didn't provoke bacterial reverse mutations, chromosomal aberrations, and micronucleated reticulocytes. Therefore, freeze-dried *P. brevitarsis* larva powder shows no evidence of toxic and mutagenic changes under the experimental conditions of the present in vitro and in vivo studies.

1. Introduction

White-spotted flower chafer (*Protaetia brevitarsis*) is a large beetle belonging to the subfamily Cetoniinae that inhabits East Asia and Europe. Dried or fried *P. brevitarsis* larvae were used widely as a traditional Asian medicine for hepatic disorders, and some studies actually have confirmed that they show liver-protective effects in mice and rats [1,2]. Furthermore, peptides purified from *P. brevitarsis* larva have shown antibacterial activities against gram-negative and gram-positive bacteria [3], while the extracts have shown antioxidant activities [4].

It has been proven that there are rich and excellent sources of important nutrients in *P. brevitarsis* larva. In a nutritional composition analysis of *P. brevitarsis* larva powder, the content of crude protein, fat, fiber, and ash from the third instar larval stage is 58%, 17%, 5%, and 8%, respectively, and it has 18% essential and 34% non-essential amino acids. Moreover, *P. brevitarsis* larva contains 61% unsaturated fatty acids as oleic acid among all fatty acids [5]. On this basis, *P. brevitarsis* larva shows promise as a novel food source providing positive nutritional benefits and attracts many scientists to identify the potential efficacy and safety of *P. brevitarsis* larva. Also, to be recognized as a food source, it is necessary to conduct the genotoxicity and acute and subchronic toxicity assessments required by the Korean government.

A 28-day oral repeated dose toxicity study of *P. brevitarsis* larva powder in Sprague-Dawley (SD) rats showed a lower body weight gain in females of the low and middle-dose groups, but no evidence of adverse changes after four weeks of oral gavage up to doses of 3000 mg/kg/day, the maximum feasible dose [6]. However, since the study was conducted to inform dose selection for subsequent 13-week study, there is still insufficient background information on the genotoxicity and oral toxicity of *P. brevitarsis* larva after long-term exposures. In terms of their promising prospects in supporting human health, additional reliable toxicity investigations are warranted to ensure their safety.

Therefore, the objective of this study was to characterize the single-dose and 13-week oral repeated dose toxicity of *P. brevitarsis* larva powder in SD rats and evaluate their genotoxicity based on the in vitro chromosome aberration test, in vivo micronucleus test, and bacterial

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Table 1
Poisoning pathogen analysis of *P. brevitarsis* larva powder.

Hazardous substance	Content	Results
Heavy metals[a]	Mercury (Hg)	0.1 ± 0.042 mg/kg
	Arsenic (AS)	Not detected
	Cadmium (Cd)	Not detected
Food poisoning bacteria[b]	*Escherichia coli* (O157:H7)	Not detected
	Salmonella spp.	Not detected
	Aerobic plate count	Not detected
Fungi[c]	*Candida albicans*	Not detected
	Saccharomyces cerevisiae	Not detected
	Aspergillus niger	Not detected

[a] Maesured by inductively coupled plasma optical emission spectrometer (IC-OES, Horiba, Japan).
[b] Incubated in Luria-Bertani (LB, Sigma-Aldrich Co.) agar media at 37 °C for 24 h.
[c] Incubated in Potato Dextrose Agar (PDA, Difco, Franklin Lakes, NJ, USA) agar media at 37 °C for 48 h.

reverse mutation test, as part of the preclinical toxicity program to develop a novel food material.

The toxicity studies were conducted in accordance with the Good Laboratory Practice of the Korean Ministry of Food and Drug Safety (MFDS) and the Organization for Economic Cooperation and Development (OECD). All studies were conducted under the MFDS and OECD test guidelines. The animal procedures were reviewed by the Institutional Animal Care and Use Committee of the Korea Institute of Toxicology, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2. Materials and methods

2.1. Materials

The third instar larval stage of *P. brevitarsis* larvae (Smurf Bugs Country Farm, Republic of Korea) was freeze-dried at −80 °C, ground into powder, and sterilized at 115 °C and 0.9 kgf/cm^2 for 5 min by World Way Co. (Republic of Korea). The powder was absent of food poisoning pathogens including heavy metals, bacteria, and fungi (Table 1) except for a small amount of mercury (0.1 ± 0.042 mg/kg) which complied with the standards and specifications of the Korean Food Standards Codex [5,7]. The general components of *P. brevitarsis* larva powder are presented in Table 2.

2.2. Formulation and analysis

P. brevitarsis larva powder was suspended in distilled water to reach the target concentrations. The dosing formulations were prepared daily and continuously stirred by magnetic stirrer. For a concentration analysis, oleic acid was chosen as a representative marker since it was the primary fatty acid in the larva of *P. brevitarsis* [8]. In the analysis results using gas chromatography (GC-2010 Plus, Shimadzu, Japan), the formulations in the range 30–300 mg/mL have shown to be homogeneous and stable for at least 24 h at room temperature and 3 days at refrigeration. The concentrations of the dosing formulations were 100.0–105.2% and 98.8–100.6% on the dosing start date (Day 1) and last week (Week 13), respectively.

Table 2
General components of *P. brevitarsis* larva powder.

General component	Composition (%)	
Moisture	6.66	± 6.40
Crude protein	57.86	± 0.01
Crude fat	16.57	± 1.81
Crude ash	8.36	± 0.10
Crude fiber	5.31	± 0.10
Total Carbohydrate[a]	10.56	± 4.49

Each value represents the mean ± standard deviation.
[a] Total Carbohydrate: 100 – sum of moisture, crude protein, crude fat and crude ash.

2.3. Animals and maintenance

Specific pathogen-free SD rats (Crl:CD) and ICR mice (CrljOri:CD1) were purchased from Orient Bio Co. (Republic of Korea). Rats and mice were housed 2 animals per cage in stainless steel wire cages and polycarbonate cages with aspen bedding, respectively. Sterilized tap water and rodent pellet diet (PMI Nutrition International, USA) were supplied ad libitum. The animals were housed in appropriate environmental conditions (22 ± 3 °C, 30–70% relative humidity, 12-h light/dark cycle at 150–300 lux, 10–20 air ventilations/h). All procedures in this study were in compliance with the Animal Welfare Act and Guide for the Care and Use of Laboratory Animals by the Institute for Laboratory Animal Research.

2.4. Single-dose oral toxicity study

2.4.1. Experimental design

This study was designed to evaluate a maximum tolerated dose (MTD) determined by acute toxicity study in accordance with OECD Guideline No. 423 (December 17, 2001). As per the guideline, three female rats aged eight weeks were selected for the test group receiving a single dose of 5000 mg/kg. Following about 17 h of fasting, the animals received *P. brevitarsis* larva powder once orally (20 mL/kg). The endpoints included clinical signs, body weight change, and macroscopic observations.

2.4.2. Clinical observation and body weight

All test animals were observed clinically five times on the dosing day (pre-dosing, 0.5, 1, 2, 3, and 4 h post-dosing) and daily thereafter for a total of 15 days. Individual body weight measurements were performed on days 1, 2, 4, 8, and 15.

2.4.3. Gross findings

A complete necropsy examination was performed after anesthesia by CO_2 inhalation and euthanasia by exsanguination on all animals.

2.5. 13-week oral repeated dose toxicity study

2.5.1. Experimental design

This study was conducted to investigate potential subchronic toxicities including hypersensitivity. Male and female rats aged six weeks were assigned to one of four groups (ten animals/sex/group) at doses of 0, 300, 1000, or 3000 mg/kg/day. The high dose was selected based on the ICH guideline M3(R2). The reversibility of any observed toxicity was assessed in a subset of additional rats (five animals/sex/group at 0 and 3000 mg/kg/day) assigned to a four-week recovery.

The doses were selected based on the results of a 28-day repeated oral administration study [6]. *P. brevitarsis* larva powder was administered orally once daily for 13 weeks (10 mL/kg) in accordance with OECD Guideline No. 408 (September 21, 1998). The endpoints included clinical observation, body weight, food consumption, ophthalmology, hematology, coagulation, clinical chemistry, urinalysis, organ weight measurement, and macro/microscopic examinations. In addition, immunoglobulin E (IgE) and histamine analyses were conducted to assess the potential allergenicity.

2.5.2. Clinical observation, body weight, food consumption, and ophthalmologic examination

Clinical observations were performed twice daily throughout the study period. In addition, detailed clinical observations were conducted

once a week for abnormal appearance and behavior. Individual body weight and food consumption were measured once weekly. Ophthalmologic examination was conducted on all animals on Week 13.

2.5.3. Clinical pathology

2.5.3.1. Hematological analysis. After termination, approximately 1.5 mL of blood was analyzed for red blood cell, hematocrit, hemoglobin, mean corpuscular hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration, reticulocyte, platelet, and white blood cell levels with an ADVIA 2120i hematology analyzer (Siemens, USA). Activated partial thromboplastin time and prothrombin time were analyzed with an ACL 9000 coagulation analyzer (Instrumentation Laboratory, Italy).

2.5.3.2. Clinical chemistry analysis. After termination, approximately 3 mL of blood was held for a minimum of 90 min at room temperature and centrifuged (3000 rpm, 10 min, room temperature) to obtain serum. Glucose, blood urea nitrogen, total protein, albumin, creatinine, albumin/globulin ratio, triglyceride, phospholipid, total cholesterol, aminotransferase, aspartate aminotransferase, and creatine kinase were analyzed with a 200FR NEO Chemistry Analyzer (Toshiba Co., Japan).

2.5.3.3. Urinalysis. Before termination, urine was collected overnight using metabolic cages. Food was withdrawn, but water was supplied. Volume, color, clarity, pH, specific gravity, urobilinogen, bilirubin, protein, nitrite, glucose, ketone, and erythrocyte levels were analyzed with a Cobas U411 urine analyzer and Combur 10 urine sticks (Roche, Germany). Moreover, microscopic examination was conducted for epithelial cell, red blood cell, white blood cell, and urine cast.

2.5.4. Gross finding, organ weight, and microscopic examination

After anesthesia with isoflurane and euthanasia by exsanguination, the complete necropsy examination was conducted on all animals, and the weights of the following organs were measured: brain, pituitary gland, liver, thymus, heart, spleen, seminal vesicles, salivary gland, prostate, adrenal glands, epididymides, testes, thyroid/parathyroid glands, lung, uterus, ovaries, and kidneys. Based on the absolute organ weight and body weight at necropsy, the relative weight (organ-to-body weight ratio) was calculated (data not shown).

All tissues from each animal were preserved with 10% neutral buffered formalin solution, except for the epididymides and testes. The epididymides and testes were fixed in Bouin's fixative for approximately 48 h and transferred to 70% ethanol. The preserved tissues were sectioned and stained with hematoxylin and eosin for the microscopic examination. The tissue slides from the vehicle control and high-dosage group were examined.

2.5.5. Allergic reaction

After termination, approximately 0.6 mL of blood for the allergic reaction analysis was collected and centrifuged (13,200 rpm, 3 min, room temperature) to obtain serum. IgE and histamine concentrations were assessed with IgE (rat) and histamine enzyme-linked immunosorbent assay (ELISA) kits (Abnova Corporation, Taiwan), respectively. The data analysis was performed with SoftMax Pro (Ver. 5.4.1; Molecular Devices Corp., USA).

2.5.6. Statistical analysis

The homogeneity of variance for body weight, food consumption, clinical pathology, histamine and IgE was analyzed with Bartlett's test. Homogeneous data were analyzed with an analysis of variance (ANOVA), and the significance of the inter-group differences was analyzed with Dunnett's test. Heterogeneous data were examined with the Kruskal-Wallis test and Dunn's rank sum test for significant differences between the control and dosing groups.

For the comparison between the control and recovery groups, the data were analyzed with the F-test for the homogeneity of variance. Homogeneous data were assessed with the t-test, and significant differences between the control and recovery groups were analyzed with Dunnett's test. Heterogeneous data were analyzed with the Kruskal-Wallis Test and Dunn's rank sum test for significant differences between the control and recovery groups. The results of the comparison were indicated when the p values were less than 0.05 or 0.01.

2.6. Genotoxicity study

2.6.1. Bacterial reverse mutation test

The purpose of this test was to evaluate the mutagenic potential of *P. brevitarsis* larva powder in four histidine auxotroph strains of *Salmonella typhimurium* and one tryptophan auxotroph strain of *Escherichia coli*. The test was carried out using the plate incorporation methods described in OECD Guideline No. 471 (July 21, 1997) and the MFDS guideline (August 24, 2012). The potential of *P. brevitarsis* larva powder to induce reverse mutations was assessed using histidine-requiring *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537 strains and the tryptophan-requiring E. coli WP2uvrA strain procured from Molecular Toxicology Inc. (USA) [9]. In the preceding dose range finding (DRF) test performed at doses of 8–5000 μg/plate, no antibacterial effects (i.e., cytotoxicity) were noted and precipitation of the test article was observed at concentrations of ≥40 μg/plate in all strains after the formulations were mixed in top agar and incubated for 48 h (data not shown).

Based on these results, at doses of 20.6, 61.7, 185.2, 555.6, 1666.7, and 5000 μg/plate, *P. brevitarsis* larva powder was incubated with the test strains in the presence or absence of a metabolic activator, S9 mixture, along with negative (distilled water) and positive controls containing 2-aminoanthrancene (2 μg/plate versus TA1535 with or without S9 mixture and 4 μg/plate versus WP2uvrA with S9 mixture), 9-aminoacridine (50 μg/plate versus TA1537 without S9 mixture), benzo(a)pyrene (2 μg/plate versus TA98 with or without S9 mixture and versus TA100 and TA1537 with S9 mixture), 2-nitrofluorene (2 μg/plate versus TA98 without S9 mixture), 4-nitroquinoline N-oxide (0.5 μg/plate versus WP2uvrA without S9 mixture), and sodium azide (0.5 μg/plate versus TA100 and TA1535 without S9 mixture) at 37 °C for 48 h, and the number of revertant colonies was counted with the naked eye.

2.6.2. In vitro chromosome aberration test in Chinese hamster lung cells

The test was conducted in Chinese hamster lung (CHL) cells (ATCC, USA) to evaluate potential structural chromosomal aberrations of *P. brevitarsis* larva powder in accordance with OECD Guideline No. 473 (July 21, 1997) and the MFDS guideline (August 24, 2012). The specific test procedure was performed based on the published methods [10,11].

In the preceding DRF test performed at doses of 19.5–5000 μg/mL, the precipitation or turbidity/precipitation of the test article were observed at all concentrations after 6 and 22 h of treatment in the absence of S9 mixture and over 78.1 μg/mL after 6 h of treatment in the presence of S9 mixture at the beginning and end of the treatment (data not shown).

Based on the DRF results, high doses of the main test were selected at 80 μg/mL in the presence of S9 mixture and 20 μg/mL in the absence of S9 mixture over short (6 h) and continuous (24 h) treatments. After air-drying and Giemsa-staining in Gurr buffer, slides with CHL cells were microscopically examined according to the JEMS-MMS [12]. Chromosomal aberrations were evaluated in 100 well-spread metaphases, each containing 23–27 chromosomes, and additional 100 metaphases were examined to determine the frequency of diploid (DP), polyploid (PP, ≥37 chromosomes), and endoreduplication (ER). The slide scoring results were expressed as the number of aberrant metaphases and total aberrations/100 metaphases.

2.6.3. In vivo micronucleus test in mice

The in vivo micronucleus test was conducted to investigate micronucleus caused by a consequence of chromosomal damage in accordance with OECD Guideline No. 474 (July 21, 1997) and the MFDS guideline (August 24, 2012). *P. brevitarsis* larva powder was administrated orally once daily for two days to male and female mice aged seven weeks at doses of 0, 500, 1000, and 2000 mg/kg/day (6 animals/sex/group, 10 mL/kg). In addition, cyclophosphamide monohydrate (CPA) was administrated intraperitoneally once to a positive control group at 70 mg/kg. After anesthesia by CO_2 inhalation and euthanasia by exsanguination of all animals 24 h after the final administration, bone marrow slides were prepared according to the method of Schmid [13]. A total of 2000 polychromatic erythrocytes (PCEs) per each animal were scored to evaluate the frequency of micronucleated polychromatic erythrocytes (MNPCEs). As an indicator of cytotoxicity, the PCE/(PCE + normochromatic erythrocyte [NCE]) ratio was calculated by counting 500 erythrocytes per each animal.

2.6.4. Statistical analysis

The statistical analyses for the in vitro chromosomal aberration test, according to the published statistical procedure [14], were conducted in SAS/STAT software (Ver. 9.2 SAS; Institute Inc., USA). The frequency of PP + ER and the number of aberrant metaphases (excluding gaps) were evaluated with χ^2-test and Fisher's exact test. The statistical evaluation of the in vivo micronucleus test was performed according to the published statistical procedure [15]. The number of MNPCEs between the control and dosing groups was evaluated with Kruskal-Wallis H-test and Dunn's Rank Sum test. The PCEs/(PCEs + NCEs) ratio between the control and dosing groups was evaluated by ANOVA test and Dunnett's test. The changes in body weight were analyzed with Bartlett's test and one-way parametric ANOVA. The results were considered statistically significant when the p value was less than 0.05. No statistical analysis was conducted in the bacterial reverse mutation test.

3. Results

3.1. Single-dose oral toxicity study

3.1.1. Clinical observations and body weight

No mortality or signs of toxicity were observed during the study.

3.1.2. Gross findings

No test article-related gross findings were observed in any animal.

3.2. 13-week oral repeated dose toxicity study

3.2.1. Clinical observation, body weight, food consumption, and ophthalmologic examination

No mortality or obvious clinical signs related to the test article were observed in any animal. Body weight and food consumption showed no significant differences between the control and dosing groups. No abnormalities were found in either sex of any group in the ophthalmologic examination. The body weight changes are presented in Fig. 1.

3.2.2. Clinical pathology

No test article-related changes in clinical pathology were observed in any group. In the hematology results, a statistically significant decrease in APTT and increase in PT were noted in male and female at 3000 mg/kg/day on Week 13 ($p < 0.01$) respectively. These changes were considered of little toxicological significance since they were no microscopic correlates and marked the normal physiological ranges [16]. In the clinical chemistry results, statistically significant changes in CREA, TG, and PL noted in males or females were also considered incidental as they were inconsistent between sexes and showed no dose-related patterns. The hematology and clinical chemistry values are presented in Tables 3 and 4, respectively.

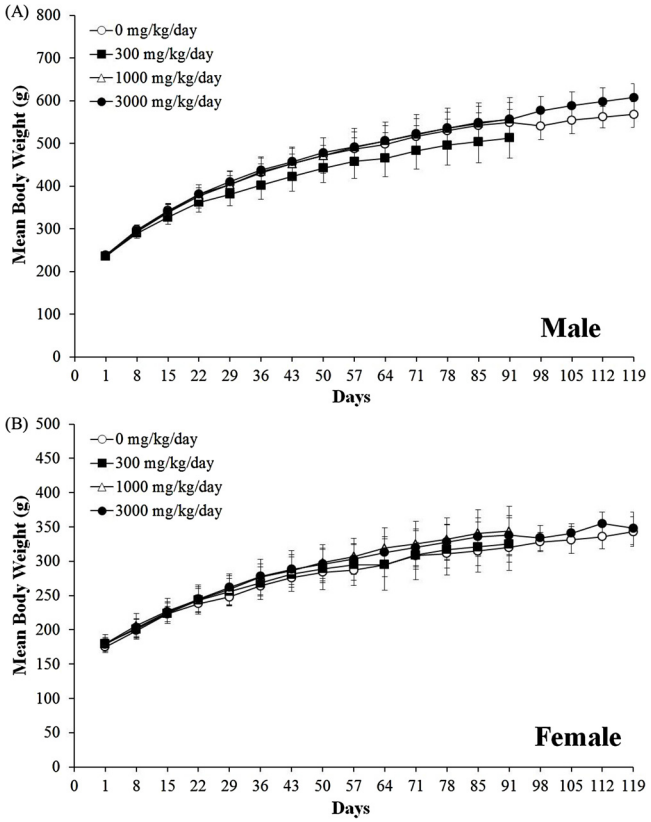

Fig. 1. Mean body weights of male (A) and female (B) rats administered *P. brevitarsis* larva powder orally.

3.2.3. Gross findings, organ weight, and microscopic examination

No test article-related changes were observed in the weight of any organ. In the macroscopic or microscopic findings, no test article-related lesions were observed in any group (data not shown). Some microscopic findings including cortical vacuolation of the adrenal glands or infiltration of mononuclear cell into several organs were observed, but they were infrequent, generally of low severity, and similarly distributed among control and dosing groups. The absolute organ weights are presented in Table 5.

For the absolute organ weights, statistically significant increases in heart weight were noted in females with a dose-dependent pattern on Week 13 ($p < 0.05$), but the change was not considered toxicologically significant since there were no clinical-pathological changes associated with heart disorder and no histopathologic findings in heart. A statistically significant decrease in thyroid/parathyroid weight in males at 300 mg/kg/day was considered incidental since there was no dose-dependence and microscopic abnormality. The microscopic character of heart and thyroid was presented in Fig. 2.

3.2.4. Allergic reaction

There were no significant changes in histamine concentrations in either sex of any group. A statistically significant increase in IgE concentrations in males at 300 mg/kg/day was observed, but the change was considered incidental since there was no dose-dependent and the concentration was close to basal level of total IgE in rats (≤30 ng/mL) [17] (Table 6).

3.3. Genotoxicity study

3.3.1. Bacterial reverse mutation test

There were no increases in the number of revertant colonies in the histidine-requiring S. typhimurium strains (TA98, TA100, and TA1537) and the E. coli strain (WP2uvrA) at any concentration compared to the

Table 3
Hematology values of rats administered *P. brevitarsis* larva powder orally for 13 weeks.

Parameters	Vehicle control		300 mg/kg/day		1000 mg/kg/day		3000 mg/kg/day	
Males								
WBC ($\times 10^3$/μL)	9.27	± 1.763	8.75	± 1.421	10.06	± 2.304	10.26	± 2.554
RBC ($\times 10^6$/μL)	9.21	± 0.306	9.24	± 0.487	9.37	± 0.313	9.14	± 0.372
HGB (g/dL)	15.9	± 0.47	16.3	± 0.63	16.1	± 0.41	16.1	± 0.60
HCT (%)	49.5	± 1.58	50.5	± 2.47	50.2	± 1.48	50.3	± 2.16
MCV (fl)	53.8	± 1.25	54.7	± 1.51	53.5	± 1.05	55.0	± 1.58
MCH (pg)	17.3	± 0.36	17.7	± 0.62	17.2	± 0.41	17.6	± 0.62
MCHC (g/dL)	32.1	± 0.43	32.3	± 0.63	32.1	± 0.43	32.0	± 0.59
PLT ($\times 10^3$/μL)	1014	± 216.2	1096	± 151.9	1078	± 157.5	1107	± 145.7
RET% (%)	2.28	± 0.379	2.04	± 0.375	2.12	± .0240	2.12	± 0.298
PT (sec)	14.6	± 0.82	14.3	± 0.74	13.9	± 0.47	15.1	± 0.79
APTT (sec)	18.5	± 1.67	17.1	± 2.84	17.7	± 0.74	15.7	± 1.22[+]
Females								
WBC ($\times 10^3$/μL)	7.52	± 2.048	6.66	± 1.743	7.57	± 2.815	7.81	± 2.113
RBC ($\times 10^6$/μL)	8.72	± 0.338	8.58	± 0.379	8.36	± 0.474	8.42	± 0.252
HGB (g/dL)	16.2	± 0.58	15.6	± 0.47	15.8	± 0.88	15.5	± 0.42
HCT (%)	49.5	± 1.51	48.0	± 1.37	48.2	± 2.95	47.6	± 1.10
MCV (fl)	56.8	± 1.46	55.9	± 0.96	57.7	± 1.99	56.6	± 1.35
MCH (pg)	18.7	± 0.54	18.2	± 0.43	18.9	± 0.51	18.5	± 0.45
MCHC (g/dL)	32.8	± 0.48	32.6	± 0.39	32.8	± 0.44	32.6	± 0.27
PLT ($\times 10^3$/μl)	1003	± 95.6	1050	± 166.1	965	± 174.0	973	± 100.4
RET% (%)	2.33	± 0.308	2.19	± 0.352	2.52	± 0.553	2.27	± 0.351
PT (sec)	14.3	± 0.54	14.0	± 0.37	14.5	± 0.37	15.0	± 0.49[+]
APTT (sec)	16.2	± 1.77	15.6	± 1.30	15.0	± 1.87	15.6	± 2.05

Each value represents the mean ± standard deviation.
WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet; RET, reticulocyte; PT, prothrombin time; APTT, activated partial thromboplastin time.
[+] Significant differences from control group ($p < 0.01$).

vehicle control in either the presence or absence of S9 mixture. In S. typhimurium TA1535, the number of revertant colonies increased by 1.9 times at 5000 μg/plate compared to the vehicle control, but did not meet the criterion for a positive response (> 3-fold). On the other hand, the number of revertant colonies from the positive controls increased more than three times in the presence and absence of S9 mixture. The precipitation of the test article was observed at concentrations ≥61.7 μg/plate. The results of the reverse mutation test are presented

Table 4
Clinical chemistry values of rats administered *P. brevitarsis* larva powder orally for 13 weeks.

Parameters	Vehicle control		300 mg/kg/day		1000 mg/kg/day		3000 mg/kg/day	
Males								
GLU (mg/dL)	106.8	± 16.27	104.3	± 15.07	109.5	± 17.10	120.3	± 24.09
BUN (mg/dL)	15.8	± 2.03	15.8	± 1.88	15.3	± 1.91	15.0	± 1.99
CREA (mg/L)	0.53	± 0.075	0.42	± 0.049[+]	0.44	± 0.059[+]	0.42	± 0.052[+]
TP (g/dL)	6.98	± 0.211	6.85	± 0.307	7.05	± 0.399	6.83	± 0.304
ALB (g/dL)	4.38	± 0.094	4.29	± 0.137	4.37	± 0.130	4.27	± 0.145
A/G (ratio)	1.70	± 0.142	1.68	± 0.136	1.65	± 0.173	1.68	± 0.106
AST (IU/L)	122.0	± 17.27	123.5	± 12.57	140.9	± 13.34	128.7	± 22.28
ALT (IU/L)	34.3	± 7.86	36.2	± 14.12	29.5	± 3.42	31.1	± 5.17
CK (IU/L)	579	± 189.2	601	± 181.3	747	± 181.9	644	± 199.3
TCHO (mg/dL)	63.3	± 17.37	54.0	± 11.38	65.1	± 20.31	57.7	± 12.72
TG (mg/dL)	32.8	± 11.10	31.0	± 6.07	54.5	± 27.26[*]	37.0	± 11.44
PL (mg/dL)	95	± 18.6	85	± 13.0	100	± 24.5	89	± 15.0
Females								
GLU (mg/dL)	119.6	± 31.11	127.0	± 40.30	130.6	± 29.09	151.7	± 30.28
BUN (mg/dL)	16.2	± 2.88	17.9	± 2.38	18.1	± 3.57	16.2	± 3.10
CREA (mg/L)	0.53	± 0.052	0.58	± 0.085	0.55	± 0.088	0.53	± 0.081
TP (g/dL)	7.33	± 0.318	7.50	± 0.389	7.46	± 0.424	7.60	± 0.573
ALB (g/dL)	4.72	± 0.210	4.81	± 0.230	4.82	± 0.202	4.83	± 0.317
A/G (ratio)	1.82	± 0.121	1.80	± 0.111	1.83	± 0.105	1.758	± 0.130
AST (IU/L)	122.6	± 23.23	118.5	± 27.54	112.1	± 17.92	118.0	± 24.30
ALT (IU/L)	30.8	± 8.95	39.5	± 16.87	39.1	± 12.13	47.7	± 33.12
CK (IU/L)	550	± 158.6	482	± 162.9	439	± 118.1	443	± 187.6
TCHO (mg/dL)	69.9	± 9.42	83.1	± 16.66	68.9	± 19.53	84.0	± 12.72
TG (mg/dL)	33.2	± 4.91	40.1	± 12.42	37.9	± 6.14	34.0	± 5.79
PL (mg/dL)	132	± 19.2	157	± 25.4	138	± 27.3	159	± 22.1[*]

Each value represents the mean ± standard deviation.
GLU, glucose; BUN, blood urea nitrogen; CREA, creatinine; TP, total protein; ALB, albumin; A/G, albumin/globulin ratio; AST, aspartate aminotransferase; ALT, aminotransferase; CK, creatine kinase; TCHO, total cholesterol; TG, triglyceride; PL, phospholipid.
[*] Significant differences from control group ($p < 0.05$).
[+] Significant differences from control group ($p < 0.01$).

Table 5
Organ weight values of rats administered *P. brevitarsis* larva powder orally for 13 weeks.

Parameters	Vehicle control	300 mg/kg/day	1000 mg/kg/day	3000 mg/kg/day
Males				
Liver (g)	14.070 ± 1.1973	12.483 ± 1.3697	14.405 ± 1.8809	13.875 ± 1.4132
Pituitary gland (g)	0.012 ± 0.0020	0.012 ± 0.0014	0.011 ± 0.0016	0.013 ± 0.0022
Spleen (g)	0.885 ± 0.0925	0.767 ± 0.1341	0.812 ± 0.1008	0.895 ± 0.1960
Heart (g)	1.548 ± 0.0903	1.395 ± 0.1240	1.496 ± 0.1108	1.443 ± 0.2196
Thymus (g)	0.390 ± 0.0554	0.392 ± 0.0841	0.417 ± 0.1081	0.399 ± 0.0760
Kidneys (g)	3.733 ± 0.3718	3.483 ± 0.3028	3.483 ± 0.3280	3.779 ± 0.3030
Lung (g)	1.653 ± 0.1477	1.668 ± 0.1481	1.671 ± 0.1256	1.733 ± 0.1810
Adrenal gland (g)	0.066 ± 0.0078	0.063 ± 0.0081	0.062 ± 0.0091	0.066 ± 0.0083
Thyroid/parathyroid (g)	0.027 ± 0.0028	0.023 ± 0.0027*	0.025 ± 0.0030	0.029 ± 0.0048
Females				
Liver (g)	7.899 ± 0.6012	8.264 ± 0.9282	8.667 ± 1.1801	8.662 ± 0.5968
Pituitary gland (g)	0.017 ± 0.0034	0.016 ± 0.0019	0.016 ± 0.0037	0.016 ± 0.0036
Spleen (g)	0.561 ± 0.0512	0.529 ± 0.0700	0.612 ± 0.1086	0.590 ± 0.0635
Heart (g)	0.975 ± 0.0852	0.993 ± 0.0830	1.077 ± 0.0950*	1.089 ± 0.0880*
Thymus (g)	0.289 ± 0.0663	0.325 ± 0.0546	0.348 ± 0.0754	0.354 ± 0.0696
Kidneys (g)	2.131 ± 0.1378	2.097 ± 0.2218	2.196 ± 0.2318	2.218 ± 0.1210
Lung (g)	1.263 ± 0.1373	1.230 ± 0.1028	1.362 ± 0.0740	1.343 ± 0.1195
Adrenal gland (g)	0.081 ± 0.0103	0.078 ± 0.0052	0.080 ± 0.0166	0.081 ± 0.0070
Thyroid/parathyroid (g)	0.019 ± 0.0037	0.019 ± 0.0024	0.020 ± 0.0057	0.020 ± 0.0031

Each value represents the mean ± standard deviation.
* Significant differences from control group ($p < 0.05$).

in Table 7.

3.3.2. *In vitro chromosome aberration test in CHL cells*

P. brevitarsis larva powder did not induce any significant increase in the frequency of metaphases with chromosomal aberrations at all concentrations after 6 and 22 h of treatment in either the presence or absence of S9 mixture (Table 8). The number of aberrant metaphases in the positive control increased with statistical significance, supporting

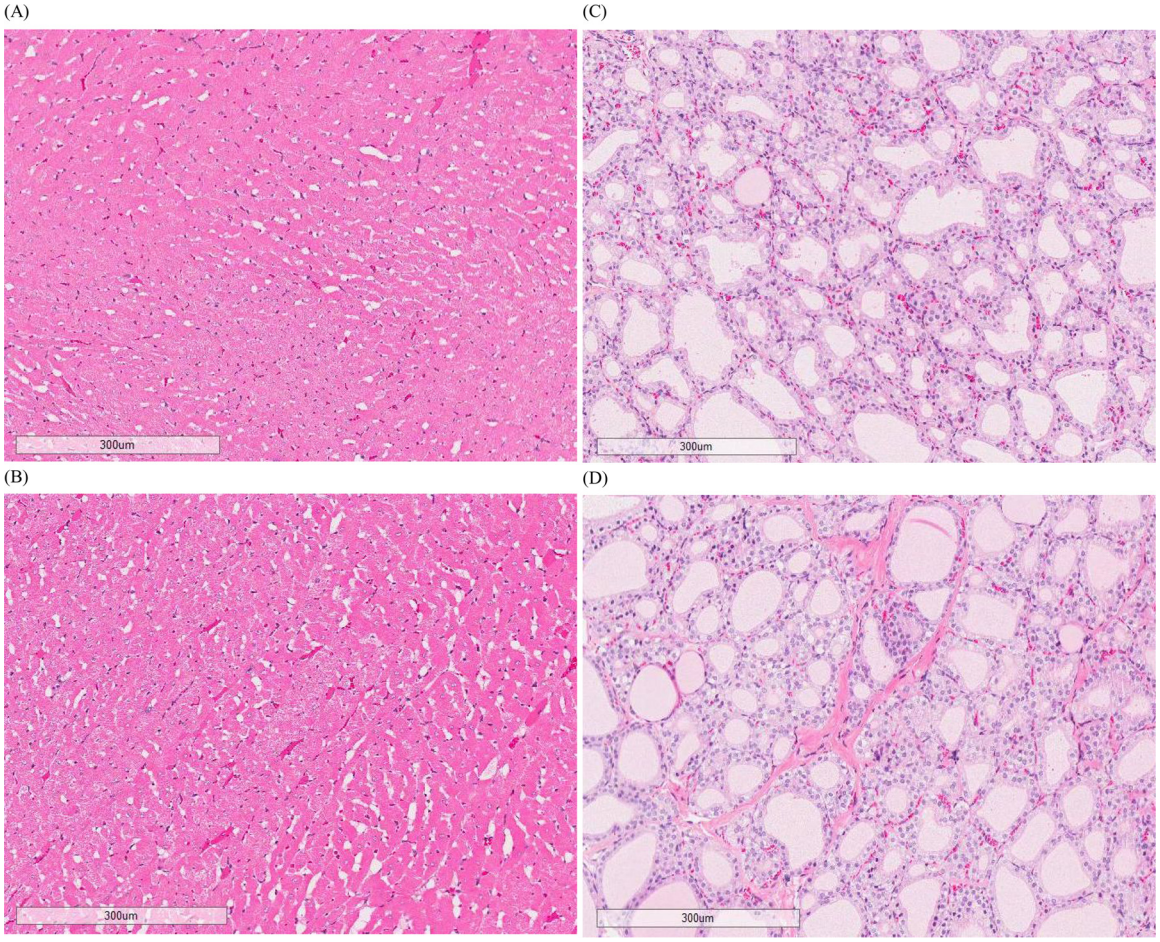

Fig. 2. Selected microphotographs of heart and thyroid from rats administered larva powder orally for 13 weeks. (A) Control heart; (B) Heart of 3000 mg/kg/day; (C) Control thyroid; (D) Thyroid of 3000 mg/kg/day.

Table 6
Serum IgE and histamine concentrations in male and female SD rats orally administered *P. brevitarsis* larva powder.

Sex	Dose (mg/kg/day)	IgE (ng/mL)				Histamine (ng/mL)			
		Dosing group		Recovery group		Dosing group		Recovery group	
No. of Animals		10		5		10		5	
Males	0	9.3	± 4.0	19.8	± 9.2	86.3	± 34.1	101.8	± 13.8
	300	38.3	± 33.8[+]	NA		101.2	± 24.6	NA	
	1000	15.3	± 8.8	NA		113.6	± 36.2	NA	
	3000	19.3	± 11.9	17.9	± 11.5	136.7	± 62.2	121.3	± 78.0
Females	0	29.8	± 34.6	27.5	± 12.4	100.7	± 35.6	87.2	± 15.0
	300	16.4	± 14.7	NA		111.3	± 48.0	NA	
	1000	16.4	± 11.9	NA		113.9	± 35.1	NA	
	3000	26.7	± 27.1	11.3	± 10.3	138.7	± 25.4	106.2	± 24.5

Each value represents the mean ± standard deviation.
NA, not applicable schedule; IgE, immunoglobulin E.
[+] Significant differences from control group ($p < 0.01$).

Table 7
Results of the bacterial reverse mutation test with *P. brevitarsis* larva powder.

Dose (μg/plate)		Revertant colonies/plate (Mean) [Factor][a]									
	Test strain	TA100		TA1535		TA98		TA1537		WP2uvrA	
0	Without S9 mixture	125	± 5	12	± 2	18	± 2	10	± 1	34	± 1
20.6		131	± 6 [1.0]	15	± 1 [1.3]	21	± 4 [1.2]	12	± 2 [1.2]	35	± 3 [1.0]
61.7		130	± 3 [1.0]	17	± 2 [1.4]	23	± 3 [1.3]	10	± 1 [1.0]	31	± 3 [0.9]
185.2		128	± 11[1.0]	16	± 1 [1.3]	23	± 2 [1.3]	12	± 3 [1.2]	36	± 4 [1.1]
555.6		142	± 10 [1.1]	20	± 1 [1.7]	25	± 1 [1.4]	12	± 1 [1.2]	38	± 4 [1.1]
1666.7		151	± 15 [1.2]	22	± 3 [1.8]	25	± 1 [1.4]	11	± 2 [1.1]	39	± 3 [1.1]
5000		153	± 16 [1.2]	23	± 3 [1.9]	23	± 2 [1.3]	12	± 1 [1.2]	43	± 2 [1.3]
0	With S9	131	± 10	10	± 1	32	± 1	15	± 3	37	± 2
20.6	mixture	136	± 10 [1.0]	11	± 3 [1.1]	33	± 3 [1.0]	15	± 2 [1.0]	38	± 1 [1.0]
61.7		136	± 6 [1.0]	14	± 1 [1.4]	33	± 2 [1.0]	14	± 2 [0.9]	35	± 3 [0.9]
185.2		143	± 4 [1.1]	15	± 2 [1.5]	34	± 2 [1.1]	15	± 3 [1.0]	40	± 2 [1.1]
555.6		148	± 14 [1.1]	15	± 2 [1.5]	33	± 3 [1.0]	18	± 2 [1.2]	39	± 2 [1.1]
1666.7		164	± 7 [1.3]	17	± 2 [1.7]	37	± 1 [1.2]	14	± 1 [0.9]	39	± 3 [1.1]
5000		182	± 10 [1.4]	19	± 2 [1.9]	32	± 3 [1.0]	20	± 3 [1.3]	38	± 2 [1.0]

[a] No. of revertant colonies of treated plate/No. of revertant colonies of vehicle control plate.

Table 8
Results of the chromosome aberration assay and relative cell count with *P. brevitarsis* larva powder.

Nominal concentration of test article (μg/mL)		S9 mixture	Time[a] (h)	Mean aberrant metaphases	Mean total aberrations	Mean PP + ER	Relative cell count (%)
6-h treatment							
	0	+	6-18	0.5/0.0[b]	0.5/0.0	0.5 + 0.0	100
	10	+	6-18	Not counted			95
	20	+	6-18	1.0/1.0	1.0/1.0	0.0 + 0.0	95
	40	+	6-18	0.5/0.5	2.0/2.0	0.5 + 0.0	101
	80	+	6-18	0.5/0.5	0.5/0.5	0.0 + 0.0	102
CPA	6	+	6-18	25.0/24.0[+]	31.0/29.5	1.0 + 0.0	79
6-h treatment							
	0	–	6-18	0.5/0.5	0.5/0.5	0.0 + 0.0	100
	2.5	–	6-18	Not counted			99
	5	–	6-18	0.0/0.0	0.0/0.0	0.0 + 0.0	99
	10	–	6-18	0.5/0.0	0.5/0.0	0.0 + 0.0	99
	20	–	6-18	1.0/1.0	1.0/1.0	0.5 + 0.0	95
EMS	800	–	6-18	25.0/25.0[+]	36.5/35.5	0.5 + 0.0	63
22-h treatment							
	0	–	22-2	0.5/0.5	0.5/0.5	0.5 + 0.0	100
	2.5	–	22-2	Not counted			100
	5	–	22-2	0.5/0.0	0.5/0.0	0.5 + 0.0	103
	10	–	22-2	0.5/0.5	0.5/0.5	1.0 + 0.0	100
	20	–	22-2	1.0/0.0	1.0/0.0	0.5 + 0.0	100
EMS	600	–	22-2	42.5/42.5[+]	70.0/68.5	1.0 + 0.0	57

PP, polyploid; ER, endoreduplication; CPA, cyclophosphamide monohydrate (positive control); EMS, ethyl methane sulfonate (positive control).
[a] Treatment time-recovery time.
[b] Gaps included/excluded, means of duplicate cultures; 100 metaphases were examined per culture.
[+] Significant difference from control group ($p < 0.01$).

Table 9
Results of micronucleus test with *P. brevitarsis* larva powder.

	P. brevitarsis larva powder				CPA
Dose (mg/kg)	0	500	1000	2000	70
Males					
MNPCE/2000 PCE	1.00 ± 0.89	0.83 ± 0.75	1.17 ± 0.75	1.67 ± 0.52	63.50 ± 9.73[+]
PCE/(PCE + NCE)	0.59 ± 0.07	0.61 ± 0.02	0.65 ± 0.03	0.67 ± 0.05	0.60 ± 0.05
Females					
MNPCE/2000 PCE	0.83 ± 0.41	1.17 ± 0.75	1.00 ± 0.00	1.33 ± 0.82	57.50 ± 12.53[+]
PCE/(PCE + NCE)	0.60 ± 0.04	0.63 ± 0.06	0.59 ± 0.03	0.64 ± 0.05	0.52 ± 0.04

Each value represents the mean ± standard deviation.
MNPCE, PCE with one or more micronuclei; PCE, polychromatic erythrocyte; NCE, normochromatic erythrocyte; CPA, cyclophosphamide monohydrate (positive control).
The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/SXx44T.
[+] Significant differences from control group ($p < 0.01$).

the validity of the method used in this test.

3.3.3. *In vivo* micronucleus test in mice

No mortalities, clinical signs, or significant body weight changes related to the test article were observed in either sex of any group. *P. brevitarsis* larva powder did not induce any significant increase in the proportion of MNPCEs or decrease in the PCE/(PCE + NCE) ratio in any group compared to the vehicle control group (Table 9). Significantly increased frequencies of MNPCEs in the positive control group indicated the validity of the present method.

4. Discussion

Insects represent the largest group of animals in the world, with over 700,000 species. They have essential roles in maintaining natural ecosystems due to their diversity of functions, forms, and lifestyles. Because of their nutritional and pharmacological benefits, humans have consumed insects for thousands of years, and over 1400 edible insect species have been identified worldwide [18]. Insects are increasingly highlighted as an alternate dietary food to combat dietary insufficiencies driven by the explosion in the global population. Actually various kinds of insect products including mealworm flour pasta and cricket flour are currently produced in several countries. However, the potential toxicity and food safety of insects is frequently questioned.

As an edible insect, the toxicity of freeze-dried *P. brevitarsis* larva powder was evaluated in single-dose oral toxicity, 13-week oral repeated dose toxicity, and genotoxicity tests in compliance with OECD guidelines for the testing of chemicals. The results of the single-dose oral toxicity study showed that there were no test article-related acute changes in mortality, clinical signs, or body weight. From the results of the 13-week treatment, there were no test article-related changes in mortality, clinical signs, body weight, food consumption, or ophthalmology during the study period. Some statistically significant differences observed in the clinical pathology and organ weight were considered to be incidental, as the changes were inconsistent between sexes or displayed no dose-response relationships. Moreover, all microscopic findings were considered to be unrelated to the test article or common background findings in rats [19]. In the IgE results testing for allergic reaction, only male samples showed 4.1-, 1.6-, and 2.1-fold increases at doses of 300, 1000, and 3000 mg/kg/day, respectively, compared to the vehicle control group. However, the results showed large variations among individuals (data not shown) and no dose-dependence. Females did not show increases in IgE concentrations. In the recovery group, neither males nor females showed increases in IgE concentrations.

In the histamine test, both male and female samples of the dosing groups showed slight increases (male: 1.2–1.6-fold, female: 1.1–1.4-fold), but no dose-dependence was observed. Neither male nor female samples of the recovery group showed significant increases in histamine concentrations compared to the vehicle control group. In laboratory rodents, the basal level of total IgE has been reported to be ≤30 ng/mL [17,20]. Furthermore, the basal level of histamine has been reported to be 60–120 ng/mL or less in rats or mice [21,22]. In the present study, the changes in total IgE and histamine were measured without an aberrant range against basal levels. It is unlikely that there were any effect on allergic reactions from *P. brevitarsis* larva powder in this study since previous studies have generally required the observation of large differences of up to a few hundred-fold to identify correlations between increases in IgE and histamine and allergenicity [23–25]. Since tropomyosin is known as a major cross-reactive allergen across invertebrates including insect however, the identification may be useful to check for further allergenicity test [26]. The systemic exposure measurement from further toxicokinetic analysis may also useful to estimate a correlation between the observed changes and the test item although the toxicokinetic evaluation is not required to be recognized as a food source.

The genotoxicity tests, including bacterial reverse mutation of S. typhimurium and E. coli, chromosome aberration of CHL cells, and micronucleus formation, indicated that there was no mutagenicity related to *P. brevitarsis* larva powder treatment. In the bacterial reverse mutation test, the number of revertant colonies increased in a dose-dependent manner in the presence and absence of S9 mixture in the S. typhimurium TA1535 strain, which reverted from auxotrophy to prototrophy via base substitution mutations; however, this was just lower than 2 times increase and also did not meet the criterion of a positive result, defined as an increase of over 3-fold [27]. There were no increases in the number of revertant colonies in other strains tested.

5. Conclusions

The results of the single-dose oral toxicity and 13-week oral repeated dose toxicity experiments suggest that the maximum tolerated dose of test samples (MTD) of freeze-dried powder of *P. brevitarsis* larva in rats is greater than 5000 mg/kg and that the no-observed-adverse-effect level (NOAEL) is 3000 mg/kg/day in rats, the highest dose tested. The results of the genotoxicity tests indicate that freeze-dried *P. brevitarsis* larva powder is not genotoxic. Therefore, freeze-dried *P. brevitarsis* larva powder shows no evidence of toxicity under the present experimental conditions.

Conflicts of interest

There are no competing financial interests.

Transparency document

The Transparency document associated with this article can be found in the online version.

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