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- Food Science of Animal Resources -
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Article Title	Subacute Oral Toxicity Evaluation of Expanded-Polystyrene-Fed <i>Tenebrio molitor</i> Larvae (Yellow Mealworm) Powder in Sprague-Dawley Rats
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10 *Tenebrio molitor* larva, as known as edible insects, has advantages of being rich in protein,
11 and has been recognized as a suitable alternate protein source for broiler and pig feed.
12 Moreover, given their ability to biodegrade polystyrene, a major pollutant, *Tenebrio molitor*
13 larvae has been proposed as an innovative solution to environmental problems. In the present
14 study, we investigated the toxicity of *Tenebrio molitor* larvae powder (TMlp) ingested with
15 expanded-polystyrene (W/ eps) through *in vitro* and *in vivo* experiments. The objective of this
16 study was to determine whether TMlp W/ eps can be applied as livestock alternative protein
17 source. For *in vitro* experiments, cytotoxicity test was performed to investigate the effects of
18 TMlp-extract on the viability of estrogen-dependent MCF-7 cells. The possibility of estrogen
19 response was investigated in two groups: expanded-polystyrene-fed TMlp (W/ eps) group and
20 without expanded-polystyrene-fed TMlp (W/o eps) group. For *in vivo* experiments, The male
21 Sprague-Dawley (SD) rats were divided based on the dosage of TMlp administered and oral
22 administration was performed to every day for 5 weeks. A toxicological assessments were
23 performed, which included clinical signs, food consumption, body and organ weights,
24 hematology, serum chemistry, and hematoxylin and eosin staining of liver and kidney. There
25 were no specific adverse effect of TMlp W/ eps-related findings under the experimental
26 conditions of this study, but further studies on both sexes and animal species differences
27 should be investigated. In conclusion, TMlp W/ eps was considered non-toxic and observed to
28 be applicable as an alternative protein source for livestock feed.

29 Keywords: *Tenebrio molitor*, expanded polystyrene, edible insect, food safety, subacute
30 toxicity

31

32 Introduction

33 Recently, with increase in world population, problems related to environmental pollution as

34 well as food supply and demand due to industrialization have been rising (Van et al., 2013a).
35 To respond to these changes in the food production industry, edible insects are attracting
36 attention as an innovative protein source. In 2013, United Nations' Food and Agriculture
37 Organization (FAO) designated insects as a future food resource (Lee et al., 2017; Van,
38 2013b). Edible insects have a short growing period and generate a small amount of biological
39 waste, thus serving as an eco-friendly and economical future food resource (Ooninx and
40 Boer, 2012; Yun and Hwang, 2016). In addition, it contains more essential amino acids than
41 soybean, indicating their great potential for use as an alternative to plant protein (Yi et al.,
42 2013). In particular, edible insects are highly regarded for their nutritional value because they
43 can supply amino acids lacking in the living body and contain abundant nutrients, such as
44 unsaturated fatty acids and minerals (Park and Choi, 2020; Yun and Hwang, 2021). Moreover,
45 they are suitable feed ingredients as an alternative protein source for broiler (Biasato et al.,
46 2018; Bovera et al., 2016; De et al., 2015; Elorduy et al., 2002) and pigs (Cho et al., 2019; Jin
47 et al., 2016; Yoo et al., 2019). Accordingly, a number of studies are underway worldwide,
48 aimed at exploring the application of edible insects as an important alternative food for
49 protein supply to livestock.

50 *Tenebrio molitor* larvae (Yellow mealworm), belonging to the Tenebrionidae family of the
51 Coleoptera order is one of the most common edible insects. These insects exhibit a strong
52 adaptability to harsh environments, such as drought and temperature (high and low), and are
53 considered among the edible insects that can be readily industrialized due to the ease of mass
54 breeding. These insects need not be bred at a large scale and are widely used as a biological
55 research model due to easy handling and reproduction (Jung et al., 2014; Yang et al., 2015). In
56 addition, their safety as a raw material for food production has been determined, and the
57 European Food Safety Authority (EFSA) has validated mealworms as edible insects in 2021
58 (EFSA et al., 2021). In particular, *Tenebrio molitor* L. are protein-rich edible insects, with

59 over 50% crude protein yield, and contain abundant essential amino acids, such as threonine,
60 valine, histidine, and lysine, as well as unsaturated fatty acids and minerals, such as calcium
61 and magnesium (Baek et al., 2017; Yoo et al., 2013). The safety of *Tenebrio molitor* L.
62 powder as food has been proven through *in vivo* experiments, which revealed no obvious
63 toxicity in Sprague Dawley (SD) rats of both sexes fed $3,000 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ *Tenebrio molitor*
64 L. powder for 28 days (Han et al., 2014). In addition, in weaned pigs fed *Tenebrio molitor* L.,
65 growth performance and protein utilization were improved, and *Tenebrio molitor* L. could be
66 used as a protein source in monogastric animal (poultry and pig) feed, with comparable
67 protein quality to soybean meal (Elorduy et al., 2002; Hong et al., 2020).

68 Polystyrene (PS) is a common petroleum-based plastic produced through the
69 polymerization of styrene monomer. It is used in various packaging and building structures,
70 such as in plastic cups, packaging materials, egg trays, disposable products, and building
71 insulation (Farrelly and Shaw, 2017). In recent years, with increase in plastic consumption
72 following industrial development, a large amount of plastic waste is being generated, and due
73 to its light weight, inertness, durability, and strong structural safety, biodegradation and
74 natural decomposition of PS are extremely challenging (Gautam et al., 2007; Pushpadass et al.,
75 2010). PS is widely used in the form of expanded polystyrene (EPS) and extruded polystyrene
76 (XPS), with Styrofoam being the most common form of EPS. They were widely used in the
77 production of foam insulation and packaging materials (Matyja et al., 2020; Peng et al., 2019).
78 EPS materials are primarily manufactured for one-time use, which increases the accumulation
79 of EPS waste due to the contrasting patterns of high durability and short consumption, leading
80 to significant environmental problems (Barnes et al., 2009; Kale et al., 2015). Currently, most
81 EPS waste is disposed of in landfills or incinerated. In addition to being energy-intensive,
82 EPS incineration generates toxic substances, such as dioxins (Faravelli et al., 2001; Matyja et
83 al., 2020). Moreover, due to its structural safety, EPS is dispersed in natural systems, such as

84 soil, rivers, lakes, and seas, upon when decomposing, leading to microplastic accumulation
85 (Hidalgo et al., 2012; Wu et al., 2017). In particular, the endocrine-disrupting chemicals
86 released from these synthetic substances exhibited hormonal activity similar to estrogen, a
87 female hormone, *in vivo* and can adversely affect the human body by disrupting homeostasis,
88 reproduction, development, and behavior (Qiang et al., 2020; Swanson et al., 1995).

89 In addition to the mechanical, chemical, and thermal treatments of PS to solve these
90 problems, various efforts are underway to use edible insects, such as red flour beetles
91 (*Tribolium castaneum*) (Fabreag and Familara, 2019), superworm (*Zophobas morio* L.) larvae
92 (Yang et al., 2020), dark mealworm (*Tenebrio obscurus* L.) larvae and yellow mealworm
93 (*Tenebrio molitor* L.) larvae (Bae et al., 2021), in an environmentally friendly and economic
94 way (Maharana et al., 2007). *Tenebrio molitor* L. from 22 countries could biodegrade PS,
95 reducing its mass, and depolymerization/cleavage of long-chain structures was observed
96 (Peng et al., 2019). Furthermore, low-molecular-weight residues and functional groups
97 indicative of oxidative transformation were detected in the extract of mealworm frass (insect
98 excrement). Therefore, EPS biodegradation ability may be a universal characteristic of these
99 insects regardless of their geographical origin (Yang et al., 2018). Given their ability to alter
100 the chemical and physical properties of PS through biodegradation and mineralization,
101 *Tenebrio molitor* L. is proposed as an innovative solution to environmental problems caused
102 by the accumulation of EPS waste.

103 In this context, the application of *Tenebrio molitor* L. used for EPS biodegradation as a
104 protein source in livestock feed can serve as an economical means to mitigate the problems of
105 environmental pollution caused by the accumulation of PS waste. Moreover, it is possible to
106 stabilize protein supply and demand through edible insects in an environmentally friendly and
107 economical way. Therefore, in the present study, *in vitro* and *in vivo* toxicological safety
108 evaluations were undertaken to investigate the industrial applicability of *Tenebrio molitor* L.

109 used for EPS biodegradation as livestock feed in the powder form.

110

111 **Materials and Methods**

112 **Ethics**

113 All experimental procedures were performed in accordance with the Animal Experimental
114 Guidelines provided by the Kongju National University Institutional Animal Care and Use
115 Committee (KNU-IACUC), Korea. The experimental protocol was approved by the KNU-
116 IACUC (Approval number: KNU_2021-08).

117

118 **Cell culture**

119 The breast cancer cell line MCF-7 used in the experiment was purchased in the frozen form
120 from the Korean Cell Line Bank (Seoul, Korea), thawed in a 75 cm² cell culture flask, and
121 cultured. MCF-7 cells were grown in RPMI 1640 (Welgene, Gyeongsan) containing 5% fetal
122 bovine serum (FBS; Grand Island, NY, USA) and 1% streptomycin/penicillin (Gibco BRL,
123 Grand Island, NY, USA) at 37°C under 5% CO₂. The medium was replaced every 2–3 days.
124 Upon reaching 80–90% confluency in the flask, the cell cultures were washed with
125 phosphate-buffered saline (PBS; Biosesang, Seongnam, Korea) and passaged following
126 treatment with trypsin-EDTA (Gibco, Grand Island, NY, USA). The reagents used in this
127 experiment, 17-β estradiol, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
128 (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

129

130 **Preparation of TMlp**

131 The preparation of TMlp was supported by the Inc. MCE (Daejeon, Korea). *Tenebrio molitor*
132 larva (yellow mealworm) were purchased from KEIL (Cheongju, Korea). The insects were
133 housed and bred in a dark room at 26±2°C under 65±10% relative humidity in HDPE boxes

134 (270 W × 450 L × 100 H mm). In the control group, 6–8-week-old *Tenebrio molitor* L. were
135 fed wheat bran. In the experimental group, 85% of the total intake was substituted with
136 expanded polystyrene feed blocks (EPS FBs) and the remaining 15% constituted ordinary
137 feed, such as Chinese cabbage and lettuce, to replenish moisture. In the expanded
138 polystyrene-fed experimental group, EPS FBs (bead method, type 1 insulation material no. 3)
139 were cut into 20×20×10 mm sheets weighing 0.08 g and fed to the *Tenebrio molitor* L. for 8
140 wk. Thereafter, the remaining expanded polystyrene in the intestine was completely
141 discharged through feeding only bran for 4 d. Following 2 d of fasting, TMlp was prepared
142 from 14-week-old *Tenebrio molitor* L., which is the normal age at which they are typically
143 shipped. The powder was sterilized at 120°C for 10 min using a high-pressure steam sterilizer
144 (MK-50S, Komachine, Yongin, Korea) and then heat-dried in a hot-air oven (J-300S,
145 Komachine, Yongin, Korea) at 80–85°C for 8 h. Thereafter, oil was extracted using a low-
146 temperature compressor (LOP-G3, Lequip, Seoul, Korea). The residue was pulverized
147 through a 100 mesh using a pulverizer, and the samples were stored in a –20°C freezer until
148 further use.

149

150 **Preparation of TMlp extract**

151 TMlp and 70% ethyl alcohol were leached for 3 d at a ratio of 3:7 (w/v). Then, the solution
152 was concentrated under reduced pressure at 45–50°C for 1 d using a rotary vacuum
153 concentrator (EYELA N-1000, Rikakikai, Tokyo, Japan). The concentrated samples in
154 dimethylsulfoxide (DMSO, Duksan, Seoul, Korea) were used as the final extract. The
155 concentration of the TMlp extract was selected at the high concentration of 250 mg·mL⁻¹,
156 which is the maximum dose that can be dissolved in DMSO as a solvent. Thereafter, the TMlp
157 extract was gradually diluted depending on the treatment concentration.

158

159 **Cell viability analysis**

160 Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthrazolium
161 bromide (MTT) assay. MCF-7 cells were dispensed at a density of 2×10^4 cells \cdot mL⁻¹ in a 96-
162 well plate and cultured in an incubator maintained at 37°C under 5% CO₂ for 24 h. Next, the
163 cells were treated with different concentrations of 17β-estradiol (E2) (0, 10⁻¹⁰, 10⁻⁹, 10⁻⁸, 10⁻⁷,
164 10⁻⁶, and 10⁻⁵ M). The concentration showing the greatest increase in the cell proliferation rate
165 compared to the control value was selected as the positive control group. MCF-7 cells were
166 treated with TMlp W/ eps and TMlp W/o eps extract at concentrations of 0, 50, 100, 150, 200,
167 and 250 μg \cdot mL⁻¹ and incubated for 24 h. Then, 40 μL of MTT solution dissolved in PBS at 1
168 mg \cdot mL⁻¹ was added to each well, and the cells were further incubated for 2 h. Thereafter, the
169 MTT reagent was removed, and 100 μL DMSO was added to each well to dissolve the
170 produced formazan. Finally, absorbance was measured at 595 nm using an ELISA reader
171 (Bio-Rad Laboratories, Hercules, CA, USA).

172
173 **Experimental animals**

174 All animals used in the experiment were bred in stainless steel wire mesh breeding boxes (260
175 W × 350 L × 210 H mm) in a room (A323) in the animal facility of Kongju National
176 University, Korea, at 22 ± 3°C under 30.0–70.0% relative humidity, 150–300 Lux illuminance,
177 10–15 times \cdot h⁻¹ ventilation frequency, and 12/12 h light/dark cycle. Four-week-old male
178 Sprague Dawley (SD) rats were purchased from Nara Biotech (Seoul, South Korea). Upon
179 arrival of the animals, visual inspection was performed, body weight was measured with an
180 electronic scale, and general symptoms were observed once a day during the 1-week
181 acclimatization period; during this time, normal feed and water were provided *ad libitum*
182 through a stainless steel feeder and polycarbonate bottles.

183

184 **Experimental design for oral dosage toxicity**

185 The male Sprague Dawley (SD) rats were classified following the randomized complete block
186 design based on similar average weights and stratified into a control group fed only distilled
187 water (DW) and experimental groups fed the test substance (*Tenebrio molitor* larva powder,
188 TMlp). In previous studies, *Tenebrio molitor* larva could be added up to 10% level to broiler
189 feed without negatively affecting growth performance and carcass characteristics, and could
190 completely replace SBM in broiler feed without deleterious effects. (Hong et al., 2020).

191 Therefore, referring to previous studies, the experimental group was further classified into
192 four groups based on TMlp concentration: normal- (W/o eps 10%), low-dose (W/ eps 5%),
193 middle-dose (W/ eps 10%), or high-dose concentration (W/ eps 15%) groups (n = 5 in each
194 group). The doses of TMlp administered orally were set to be 5%, 10%, and 15% of the
195 recently measured daily feed intake, and the TMlp was administered by dissolving in DW,
196 which was used as the solvent for the control group. The dosing volume, 10 mL/kg, was based
197 on the most recent body weight. Before administration, the rats fasted overnight (12 h) to
198 minimize the burden on the stomach. Drinking water was provided *ad libitum*. The TMlp was
199 administered orally once a day for 5 weeks using a syringe tube equipped with a zonde.

200

201 **Measurements of body weights and food consumption**

202 During this study period, changes in the clinical symptoms of rats were observed once a day
203 at a specific time (around 4–5 pm KST). Mortality was checked twice a day. Body weight and
204 daily food intake were measured before the start of the study and twice a week during the
205 study. To calculate daily food consumption, the food ration for each cage was measured the
206 day before the weight measurement, and the remaining amount on the day of the weight
207 measurement was measured. Food intake per rat was recalculated according to the average
208 food consumption (g/rat/day) for each animal.

209

210 **Organ weight measurements**

211 After completion of the study, the absolute and relative weights of the heart, lung, thymus,
212 liver, spleen, kidney, adrenal, and testis were measured through autopsy. In the case of
213 bilateral organs, the weights of the left and right organs were measured and the average value
214 was calculated.

215

216 **Hematological and serum biochemical analysis**

217 After completion of tests, all animals were anesthetized with ether, and blood was collected
218 from the abdominal aorta into EDTA-containing tubes. Red blood cell (RBC), white blood
219 cell (WBC), hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular
220 hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), platelet, lymphocyte,
221 neutrophil, eosinophils, basophils, monocytes, red cell distribution width (RDW), and mean
222 platelet volume (MPV) were examined for hematological testing. For serum biochemical
223 analysis, a part of the collected blood was allowed to stand at room temperature (20–22 °C)
224 for 30 min, coagulated, and then centrifuged (3,000 rpm for 30 min). Alanine transaminase
225 (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin, globulin,
226 total bilirubin (T-Bili), blood urea nitrogen (BUN), gamma glutamyl transpeptidase (γ -GTP),
227 creatinine (Crea), glucose, total protein (TP), chloride (Cl), sodium (Na), and potassium (K)
228 were measured.

229

230 **Histopathological examination**

231 After weight measurement, all organs were fixed in 10% neutral formalin solution. Following
232 sufficient fixation for at least 2 weeks, the samples were embedded using a paraffin
233 embedding machine (Tissue-Tek VIP, Sakura, Tokyo, Japan). A rotary microtome (Microm

234 HM340E, Thermo Scientific, Walldorf, Germany) was used to obtain 2–3- μm -thick sections.
235 The sections were stained with hematoxylin and eosin (H&E) and observed under an optical
236 microscope to determine toxicity.

237

238 **Statistical analysis**

239 All data are presented as mean \pm standard deviation (SD). Differences between the mean
240 values of TMlp W/ eps group and W/o eps group were analyzed by one-way analysis of
241 variance (ANOVA) to determine statistical significance. When the F-test and variance test
242 revealed equal variance (significance level = 0.05), *t*-test was performed (significance level =
243 0.05). A $p < 0.05$ was considered statistically significant.

244

245 **Results**

246 **General components of the expanded-polystyrene-fed TMlp**

247 To determine whether the composition of TMlp W/ eps was suitable for its use as a feed
248 material for livestock, a component analysis was performed. The powder contained 1.22%
249 moisture, 65.41% crude protein, 19.92% crude fat, 9.32% crude fiber, and 5.48% crude ash.
250 In particular, proteins accounted for a relatively high proportion compared with the other
251 components (Table 1).

252

253 **Viability of MCF-7 cells treated with expanded-polystyrene-fed TMlp extract**

254 The MTT assay was performed to evaluate the effects of TMlp extract with (W/ eps) and
255 without (W/o eps) expanded-polystyrene-fed on MCF-7 cell viability (Fig. 1). First, to assess
256 viability in the presence of E2, the estrogen-dependent cell line MCF-7 was treated with 0, 10^{-10} ,
257 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , and 10^{-5} M E2 for 24 h and subjected to the MTT assay. The
258 concentration maximizing the proliferation of MCF-7 cells was found to be 10^{-8} M; thus, in

259 subsequent experiments, 10^{-8} M was set as the positive control. To determine the estrogen
260 activity of W/ eps TMIp extract, MCF-7 cells were treated W/ eps TMIp extract and W/o eps
261 TMIp extract at concentrations of 0, 50, 100, 150, 200, and 250 $\mu\text{g}\cdot\text{mL}^{-1}$ and subjected to the
262 MTT assays. There were no significant differences in proliferation rate between the two
263 experimental groups and between the experimental and control groups.

264

265 **Changes in the body weight and feed intake of experimental animals**

266 To determine the effects of expanded-polystyrene-fed TMIp on the feed intake and body
267 weight of rats, the corresponding factors were measured. No specific weight loss was noted in
268 the control *versus* W/o eps 10% group and W/o eps 10% *versus* W/ eps 10% group (Fig. 2).
269 However, feed intake tended to decrease in all experimental groups on the 14th day, although
270 it gradually increased thereafter (Fig. 3). Overall, there were no significant differences in feed
271 intake between the control *versus* W/o eps 10% group and W/o eps 10% *versus* W/ eps 10%
272 group throughout the study period.

273

274 **Organ weights**

275 To determine the effects of expanded-polystyrene-fed TMIp on the *in vivo* of rats, autopsy
276 was performed after the completion of administration test to measure organ weights. The liver,
277 kidney, adrenal gland, heart, thymus, testes, lung, and spleen were extracted, and their
278 absolute (g) and relative (%) weights were measured (Table 2). The absolute weights of all
279 organs, except the heart and thymus, tended to decrease in the W/o eps 10% group compared
280 with those in the control group. However, there were no significant differences in terms of
281 relative weights except for the spleen. Moreover, There were no significant differences in
282 absolute and relative weights of all organs between the W/o eps 10% group and the W/ eps
283 10% group.

284

285 **Hematology and serum biochemistry**

286 Table 3 presents the results of hematological examination of blood collected from the
287 abdominal aorta at the time of autopsy after the completion of the study. We assessed
288 hematological differences between the TMlp W/o eps 10% group and the W/ eps 10% group.
289 Neutrophils tended to increase but lymphocytes tended to decrease in the W/ eps 10% group
290 compared with that in the W/o eps 10% group. In addition, serum components were analyzed
291 through biochemical tests, and the results are presented in Table 4. Specifically, there were no
292 significant differences between the W/o eps 10% group and the W/ eps 10% group in terms of
293 AST, ALT, and ALP, which are common indicators of liver function. Furthermore, T-Bili was
294 below the measurement range in the control, W/ eps 5%, and W/ eps 15% groups.

295

296 **H&E staining**

297 To evaluate the long-term toxicity of expanded-polystyrene-fed TMlp administration, liver
298 and kidneys were collected from rats autopsied after the end of the experiment and were
299 stained with H&E (Fig. 4). Light microscopy revealed no histopathological abnormalities in
300 the liver and kidneys of rats.

301

302 **Discussion**

303 *Tenebrio molitor* larvae (yellow mealworm) are protein-rich edible insects (crude protein
304 >50%), which are considered to be of great nutritional value as they contain abundant
305 essential amino acids, minerals, and unsaturated fatty acids (Baek et al., 2017; Yoo et al.,
306 2021). In addition, *Tenebrio molitor* L. can biodegrade expanded-polystyrene, a form of PS
307 that has been causing environmental problems in recent years, and these insects are, therefore,
308 proposed as an innovative solution to environmental problems caused by the accumulation of

309 EPS waste (Bae et al., 2021; Yang et al., 2015). Moreover, *Tenebrio molitor* L. have been
310 proven a suitable alternative protein source for broiler and pig feed (Biasato et al., 2018;
311 Bovera et al., 2016; Cho et al., 2019; De et al., 2015; Elorduy et al., 2002; Jin et al., 2016;
312 Yoo et al., 2019). Therefore, if *Tenebrio molitor* L. used for expanded-polystyrene
313 biodegradation can be applied in livestock feed, it can serve as an economic alternative
314 protein source and alleviate the problem of environmental pollution. In the present study, we
315 performed component analysis to evaluate the feasibility of using the powder of *Tenebrio*
316 *molitor* L. used for expanded-polystyrene biodegradation as a feed material for livestock.
317 Further, in order to observe the effect of this powder on the living body, toxicological safety
318 evaluations were performed through *in vitro* and *in vivo* experiments.

319 Composition analysis revealed that the expanded-polystyrene-fed TMlp contained 1.22%
320 moisture, 65.41% crude protein, 19.92% crude fat, 9.32% crude fiber, and 5.48% crude ash,
321 with proteins accounting for a relatively high proportion compared with the other components.
322 Yoo et al. (2013) reported that the yellow mealworm larvae powder contained 2.90% water,
323 50.32% crude protein, 33.70% crude fat, and 3.73% crude ash. Nam and Sim (2021) reported
324 that the Korean yellow mealworm larvae powder contained 4.10% water, 68.50% crude
325 protein, 13.50% crude fat, and 4.01% crude ash. Thus, the ratios of different components
326 show slight variations, which can be attributed to differences in the growth and manufacturing
327 environment of larvae. In the case of mycotoxins, neither total aflatoxin (the sum of B1, B2,
328 G1, and G2) nor ochratoxin A was detected, and the content of heavy metals was also trace,
329 indicating values consistent with the lowest standard for heavy metals of edible insects
330 (MFDS, 2022). Moreover, the TMlp acquired from expanded-polystyrene-fed *Tenebrio*
331 *molitor* larva contains a large amount of protein, so it can be used as a protein substitute or as
332 an economical material for other biological purposes. However, the potential toxic effects of
333 TMlp W/ eps should be further investigated in terms of animal species, sex differences, and

334 induction of allergic reactions before application to livestock feed.

335 In the *in vitro* experiment, the MTT assay was performed to observe the effect of E2 and
336 TMlp extract on MCF-7 cell viability. E2 is an estrogen receptors (ER) and exhibits
337 transcriptional activity, which is regulated by the binding of an agonist or antagonist ligand.
338 E2 affects cell growth and differentiation and is involved in the proliferation of estrogen-
339 dependent breast cancer cells (Colborn et al., 1993; Colborn et al., 1995; Harrison et al.,
340 1997). Furthermore, endocrine-disrupting substances released from synthetic substances
341 exhibit estrogen-like hormonal activity in the living body, producing adversely effects by
342 disrupting homeostasis, reproduction, development, or behavior (Swanson et al., 1995; Wu et
343 al., 2018). Therefore, in this study, E2 was used as a positive control to evaluate the validity
344 of the experiments and experiments were conducted to observe the release of estrogen, an
345 endocrine disrupting substance, from TMlp W/eps and TMlp W/o eps extracts depending on
346 whether the EPS was supplied using ER-positive cells, MCF-7 (Soule et al., 1973). First, to
347 assess the cell proliferation, MCF-7 cells were treated with E2 at concentrations of 0, 10^{-10} ,
348 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , and 10^{-5} M for 24 h and then subjected to MTT assays. The E2
349 concentration of 10^{-8} M was found to maximize the proliferation of MCF-7 cells and was set
350 as the positive control in subsequent experiments. Next, to assess the estrogen activity of W/
351 eps and W/o eps TMlp extract in MCF-7 cells, the proliferation rate was evaluated following
352 treatment. The cell viability increased after E2 treatment, confirming the validity of the
353 experiment. In addition, there were no significant differences in the cell viability and
354 proliferation rate between the W/ eps and W/o eps TMlp extract. These results suggested that
355 the W/ eps TMlp extract is non-toxic to cells and does not mediate an estrogen-induced
356 response.

357 In the *in vivo* experiment, oral toxicity test was conducted using male SD rats. No animals
358 died or showed clinical symptoms during the study period. Body weight tended to increase in

359 all experimental groups, but there was no significant differences. In the case of daily feed
360 intake, there were no significant differences between the W/o eps 10% and the W/ eps 10%
361 groups. Weight tended to decrease in all experimental groups on the 14th day, but gradually
362 increased thereafter. Acclimatization to administration may be attributed to the constant trend
363 of body weight until the 14th day. Therefore, There was no significant difference in body
364 weight and feed intake between the W/o eps 10% and the W/ eps 10% groups, which were the
365 main comparators. Five weeks after the start of administration of the test substance, autopsy
366 was performed, and the absolute (g) and relative (%) weights of organs were measured. The
367 relative weights of all organs, except the spleen, did not significantly differ between the W/o
368 eps and the control groups. In particular, the absolute weight of testis decreased significantly,
369 but there was no significant difference in its relative weights. Therefore, these trends likely
370 depend on the body weight of individual rats. However, in the case of the spleen, both
371 absolute and relative organ weight significantly differed between the W/o eps 10% and the W/
372 eps 10% groups. These results were similar to those of toxicological assays of Han et al.
373 (2016), in which lyophilized mealworm powder was administered to SD rats for 90 days.
374 Long-term weight change is a useful indicator in toxicity studies. However, the change in
375 spleen weight was weakly correlated with histopathological findings and appears to be due to
376 individual differences in physiological factors (Nirogi et al., 2014). To confirm changes in the
377 organ weight of rats fed with the W/ eps and the W/o eps TMlp, the organ weights of rats in
378 the W/o eps 10% and W/ eps 10% groups were compared, but there were no significant
379 differences. All blood and serum biochemical tests showed values within the normal range.
380 Specifically, neutrophils tended to increase but lymphocytes tended to decrease in the W/ eps
381 10% group compared with that in the W/o eps 10% group. However, in previous studies on
382 rodents, both neutrophils (10–20%) and lymphocytes (75–90%) were within the normal range
383 (Doeing et al., 2003; Mestas and Hughes, 2004). These discrepancies in analytical results may

384 be attributed to individual differences. Finally, the liver and kidneys were observed through
385 H&E staining but no histopathological abnormalities were noted. In a study by Yang et al.
386 (2015), the depolymerization/cleavage of long-chain PS (Polystyrene) structure was noted,
387 and low-molecular-weight fragments were newly formed in the stomachs of *Tenebrio molitor*
388 L.. Expanded-polystyrene is likely biodegraded by the intestinal microbes of *Tenebrio molitor*
389 L.; thus, the expanded-polystyrene-fed TMlp does not cause toxicological and pathological
390 abnormalities *in vivo*. Considering the above results, it was observed that W/ eps TMlp did
391 not cause *in vivo* toxicity in male SD rats under our experimental conditions.

392 In conclusion, our toxicological safety evaluations through *in vitro* and *in vivo* experiments
393 revealed that the W/ eps TMlp did not mediate an estrogen-induced response in MCF-7 cells
394 and did not cause toxicity in male SD rats under the experimental conditions of this study.
395 Thus, TMlp W/ eps, acquired from expanded-polystyrene-fed *Tenebrio molitor* larva, can be
396 used as an alternative protein source for livestock feed or as an economical material for other
397 biological purposes.

398

399 References

400 Bae J, Cho HW, Jung H, Park J, Yun S, Ha S, Lee Y, Kim TJ. 2021. Changes in intestinal
401 microbiota due to the expanded polystyrene diet of mealworms (*Tenebrio molitor*). *Indian J*
402 *Microbiol* 61:130–6.

403 Baek M, Hwang JS, Kim MA, Ki SH, Goo TW, Yun EY. 2017. Comparative analysis of
404 nutritional components of edible insects registered as novel foods. *Life Sci* 27:334-8.

405 Barnes DKA, Galgani F, Thompson RC, Barlaz M. 2009. Accumulation and fragmentation
406 of plastic debris in global environments. *Philos Trans R Soc B: Biol Sci* 364:1985-98.

407 Biasato I, Gasco L, De Marco M, Renna M, Rotolo L, Dabbou S, et al. 2018. Yellow
408 mealworm larvae (*Tenebrio molitor*) inclusion in diets for male broiler chickens: Effects on

409 growth performance, gut morphology, and histological findings. *Poult Sci* 97:540–8.

410 Bovera F, Loponte R, Marono S, Piccolo G, Parisi G, Iaconisi V, Gasco L, Nizza A. 2016.

411 Use of *Tenebrio molitor* larvae meal as protein source in broiler diet: Effect on growth

412 performance, nutrient digestibility, and carcass and meat traits. *J Anim Sci* 94:639-47.

413 Cho KH, Kang SW, Yoo JS, Song DK, Chung YH, Kwon GT, Kim YY. 2019. Effects of

414 mealworm (*Tenebrio molitor*) larvae hydrolysate on nutrient ileal digestibility in growing pigs

415 compared to those of defatted mealworm larvae meal, fermented poultry by-product, and

416 hydrolyzed fish soluble. *Asian-Australas J Anim Sci* 33:490–500.

417 Colborn T, Saal VFS, Soto AM. 1993. Developmental effects of endocrine-disrupting

418 chemicals in wildlife and humans. *Environ Health Perspect* 101:378-84.

419 Colborn T. 1995. Environmental estrogens: health implications for humans and wildlife.

420 *Environ Health Perspect* 103:135-6.

421 De Marco M, Martínez S, Hernandez F, Madrid J, Gai F, Rotolo L, Belforti M, Bergero D,

422 Katz H, Dabbou S, Kovitvadhi A, Zoccarato I, Gasco L, Schiavone A. 2015. Nutritional value

423 of two insect larval meals (*Tenebrio molitor* and *Hermetia illucens*) for broiler chickens:

424 Apparent nutrient digestibility, apparent ileal amino acid digestibility and apparent

425 metabolizable energy. *Anim Feed Sci Technol* 209:211–8.

426 Doeing DC, Borowicz JL, Crockett ET. 2003. Gender dimorphism in differential peripheral

427 blood leukocyte counts in mice using cardiac, tail, foot, and saphenous vein puncture methods.

428 *BMC Clin Pathol* 3:1-6.

429 EFSA Panel on Nutrition, Novel Foods and Food Allergens, Truck D, Castenmiller J,

430 Henauw SD, Hirsch-Ernst KI, Kearney J, Maciuk A, Mangelsdorf I, McArdle HJ, Naska A,

431 Pelaez C, Pentieva K, Siani A, Thies F, Tsabouri S, Vinceti M, Cubadda F, Frenzel T,

432 Heinonen M, Marchelli R, Neuhauser-Berthold M, Poulsen M, Maradona MP, Schlatter JR,

433 Loveren HV, Ververis E, Knutsen HK. 2021. Safety of dried yellow mealworm (*Tenebrio*

434 *molitor* larva) as a novel food pursuant to Regulation (EU) 2015/2283. EFSA J 19:e06343.

435 Elorduy JR, González EA, Hernández AR, Pino JM. 2002. Use of *Tenebrio molitor*
436 (Coleoptera: Tenebrionidae) to recycle organic wastes and as feed for broiler chickens. J Econ
437 Entomol 95:214–20.

438 Fabreag MAC, Familiara JA. 2019. Biodegradation of expanded polystyrene (EPS)
439 (Styrofoam) block as feedstock to *Tribolium castaneum* (Red Flour Beetle) imago: A
440 promising plastic-degrading process. WNOFNS 24:145-56.

441 Faravelli T, Pinciroli M, Pisano F, Bozzano G, Dente M, Ranzi E. 2001. Thermal
442 degradation of polystyrene. J Anal Appl Pyrolysis 60:103-21.

443 Farrelly TA, Shaw IC. 2017. Polystyrene as hazardous household waste. Household
444 Hazardous Waste Management. 1st ed. Mmerek ID (ed). IntechOpen, London, UK. pp 45–60.

445 Gautam R, Bassi AS, Yanful EK. 2007. A review of biodegradation of synthetic plastic and
446 foams. Appl Biochem Biotechnol 141:85–108.

447 Han SR, Lee BS, Jung KJ, Yu HJ, Yun EY, Hwang JS, Moon KS. 2016. Safety assessment
448 of freeze-dried powdered *Tenebrio molitor* larvae (yellow mealworm) as novel food source:
449 Evaluation of 90-day toxicity in Sprague-Dawley rats. Regul Toxicol Pharmacol 77:206–12.

450 Han SR, Yun EY, Kim JY, Hwang JS, Jeong EJ, Moon KS. 2014. Evaluation of
451 genotoxicity and 28-day oral dose toxicity on freeze-dried powder of *Tenebrio molitor* larvae
452 (Yellow Mealworm). Toxicol Res 30:121–30.

453 Harrison PTC, Holmes P, Humfrey CDN. 1997. Reproductive health in humans and
454 wildlife: are adverse trends associated with environmental chemical exposure?. Sci Total
455 Environ 205:97-106.

456 Hidalgo-Ruz V, Gutow L, Thompson RC, Thiel M. 2012. Microplastics in the marine
457 environment: A review of the methods used for identification and quantification. Environ Sci
458 Technol 46:3060–75.

459 Hong JS, Han TH, Kim YY. 2020. Mealworm (*Tenebrio molitor* larvae) as an alternative
460 protein source for monogastric animal: a review. *Animals* 10:2068.

461 Jin XH, Heo PS, Hong JS, Kim NJ, Kim YY. 2016. Supplementation of dried mealworm
462 (*Tenebrio molitor* larva) on growth performance, nutrient digestibility and blood profiles in
463 weaning pigs. *Asian-Australas J Anim Sci* 29:979–86.

464 Jung J, Heo A, Park YW, Kim YJ, Koh H, Park W. 2014. Gut microbiota of *Tenebrio*
465 *molitor* and their response to environmental change. *J Microbiol* 24:888–97.

466 Kale SK, Deshmukh AG, Dudhare MS, Patil VB. 2015. Microbial degradation of plastic: a
467 review. *J Biochem Technol* 6:952-61.

468 Lee KH, Yoon YT, Park YI, Lee HJ, Jeong NY. 2017. Quality evaluation of acorn mook
469 prepared with mealworm (*Tenebrio molitor*) Powder. *Korean J Food & Nutr* 30:1042–7.

470 Maharana T, Negi YS, Mohanty B. Recycling of polystyrene. 2007. *Polym Plast Technol*
471 *Eng* 46:729-36.

472 Matyja K, Rybak J, Hanus-Lorenz B, Wróbel M, Rutkowski R. 2020. Effects of
473 polystyrene diet on *Tenebrio molitor* larval growth, development and survival: Dynamic
474 Energy Budget (DEB) model analysis. *Environ Pollut* 264:114740.

475 Mestas J, Hughes CC. 2004. Of mice and not men: Differences between mouse and human
476 immunology. *J Immunol* 172:2731-8.

477 Ministry of Food and Drug Safety. 2022. Standards and specifications for general food.
478 Available from: https://www.foodsafetykorea.go.kr/foodcode/01_03.jsp?idx=12 Accessed at
479 Feb 4, 2022.

480 Nam HH, Sim KH. 2021. Quality characteristics of garaedduk enriched with mealworm
481 (*Tenebrio molitor*) powder 34:272-88.

482 Nirogi R, Goyal VK, Jana S, Pandey SK, Gothi A. 2014. What suits best for organ weight
483 analysis: Review of relationship between organ weight and body/brain weight for rodent

484 toxicity studies. Int J Pharm Sci Res 5:1525-32.

485 Oonincx DGAB, Boer IJMD. 2012. Environmental impact of the production of mealworms
486 as a protein source for humans-a life cycle assessment. PLoS ONE 7:e51145.

487 Park ES, Choi MK. 2020. Recognition, purchase, and consumption of edible insects in
488 Korean adults. J Nutr Health 53:190-202.

489 Peng BY, Su Y, Chen Z, Chen J, Zhou X, Benbow ME, Criddle CS, Wu WM, Zhang Y.
490 2019. Biodegradation of polystyrene by dark (*Tenebrio obscurus*) and yellow (*Tenebrio*
491 *molitor*) mealworms (Coleoptera: Tenebrionidae). Environ Sci Technol 53, 5256–65.

492 Pushpadass HA, Weber RW, Dumais JJ, Hanna MA. 2010. Biodegradation characteristics
493 of starch-polystyrene loose-fill foams in a composting medium. Bioresour 101:7258-64.

494 Qiang L, Lo LSH, Gao Y, Cheng J. 2020. Parental exposure to polystyrene microplastics at
495 environmentally relevant concentrations has negligible transgenerational effects on zebrafish
496 (*Danio rerio*). Ecotoxicol Environ Saf 206:111382.

497 Soule HD, Vazquez J, Long A, Albert S, Brennan M. 1973. A human cell line from a
498 pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 51:1409-16.

499 Swanson GM, Ratcliffe HE, Fischer LJ. 1995. Human exposure to polychlorinated
500 biphenyls (PCBs): A critical assessment of the evidence for adverse health effects. Regul
501 Toxicol Pharmacol 21:136-50.

502 Van Huis A, Van Itterbeeck J, Klunder H, Mertens E, Halloran A, Muir G, Vantomme P.
503 2013a. Edible insects: Future prospects for food and feed security. FAO UN; Report No.: 171

504 Van Huis A. 2013b. Potential of insects as food and feed in assuring food security. Annu
505 Rev Entomol 58: 563-83.

506 Wu WM, Yang J, Criddle CS. 2017. Microplastics pollution and reduction strategies. Front
507 Environ Sci Eng 11:1-4.

508 Yang SS, Wu WM, Brandon AM, Fan HQ, Receveur JP, Li Y, et al. 2018. Ubiquity of

509 polystyrene digestion and biodegradation within yellow mealworms, larvae of *Tenebrio*
510 *molitor* Linnaeus (Coleoptera: Tenebrionidae). *Chemosphere* 212:262-71.

511 Yang Y, Wang J, Xia M. 2020. Biodegradation and mineralization of polystyrene by plastic
512 eating superworms *Zophobas atratus*. *Sci Total Environ* 708:135233.

513 Yang Y, Yang J, Wu WM, Zhao J, Song Y, Gao L, et al. 2015. Biodegradation and
514 mineralization of polystyrene by plastic-eating mealworms: Part 1. Chemical and physical
515 characterization and isotopic tests. *Environ Sci Technol* 49:12080–6.

516 Yi L, Lakemond CMM, Sagis LMC, Eisner-Schadler V, van Huis A, van Boekel MAJS.
517 2013. Extraction and characterization of protein fractions from five insect species. *Food*
518 *Chem* 141:3341-8.

519 Yoo J, Hwang JS, Goo TW, Yun EY. 2013. Comparative analysis of nutritional and
520 harmful components in korean and chinese mealworms (*Tenebrio molitor*). *J Korean Soc*
521 *Food Sci Nutr* 42:249-54.

522 Yoo JS, Cho KH, Hong JS, Jang HS, Chung YH, Kwon GT, et al. 2019. Nutrient ileal
523 digestibility evaluation of dried mealworm (*Tenebrio molitor*) larvae compared to three
524 animal protein by-products in growing pigs. *Asian-Australas J Anim Sci* 32:387–94.

525 Yun EY, Hwang JS. 2016. Status and prospect for development of insect foods. *Food Sci*
526 *Ind* 49:31-9.

527 Yun EY, Hwang JS. 2021. Quality characteristics of garaedduk enriched with mealworm
528 (*Tenebrio molitor*) powder. *Korean J Food & Nutr* 34:272-88.

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530

Tables and Figures

531 **Table 1. General components of TMlp W/ eps.** TMlp, *Tenebrio molitor* larva powder; W/

532 eps, expanded-polystyrene-fed.

533

General components	Compositional average (%)
Moisture	1.22
Crude protein	65.41
Crude fat	19.92
Crude fiber	9.32
Crude ash	5.48
Mineral	Contents (mg/kg or %)
Calcium (Ca)	0.07%
Phosphorus (P)	1.22%
Potassium (K)	1.38%
Magnesium (Mg)	0.77%
Iron (Fe)	89.17
Copper (Cu)	27.97
Manganese (Mn)	24.64
Zinc (Zn)	209.49
Selenium (Se)	0.12
Mycotoxin	Contents (µg/kg)
Total aflatoxins (aflatoxin B ¹ , B ² , G ¹ , G ² and ochratoxin A)	ND ¹⁾
Heavy metal	Contents (ppm)
Lead (Pb)	0.01

Cadmium (Cd)

ND

Arsenic (As)

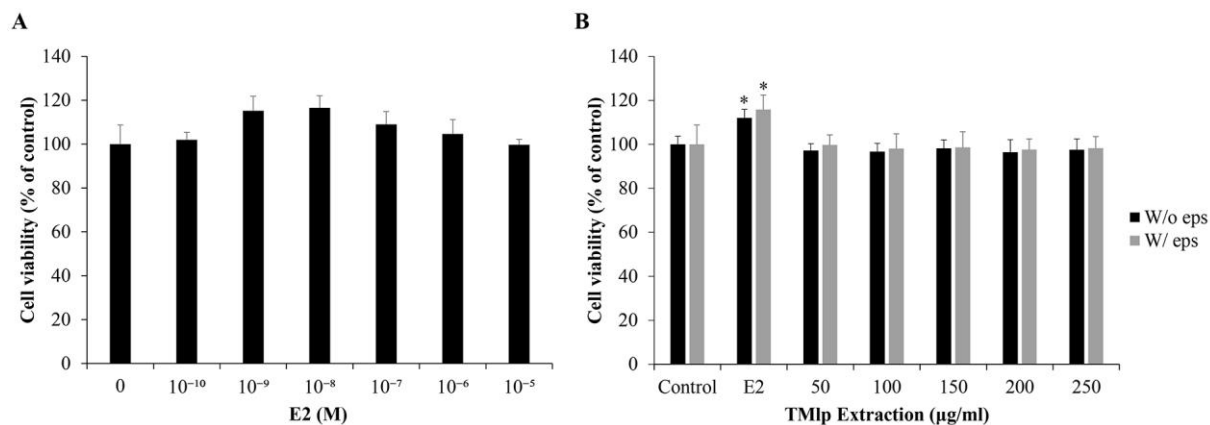
0.01

534 1) ND: Not detected.

535

536

ACCEPTED



537

538 **Fig. 1. Effects of E2 and TMlp extract on MCF-7 cell viability.** (A) Estrogen-dependent

539 MCF-7 cells were treated with various concentrations of E2 (17β-estradiol). (B) MCF-7 cells

540 were treated with various concentrations of TMlp W/o eps and W/ eps extraction. Cell

541 viability was measured by MTT assay. The results are presented as the mean±SD from three

542 independent experiments performed in triplicate. Significance was determined by Student's *t*

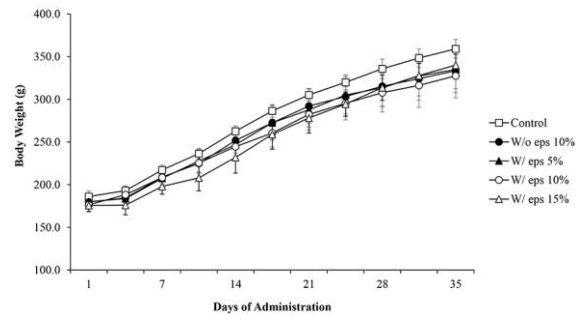
543 test, **p*<0.05 compared with control. TMlp, *Tenebrio molitor* larva powder; W/ eps,

544 expanded-polystyrene-fed; W/o eps, without expanded-polystyrene-fed.

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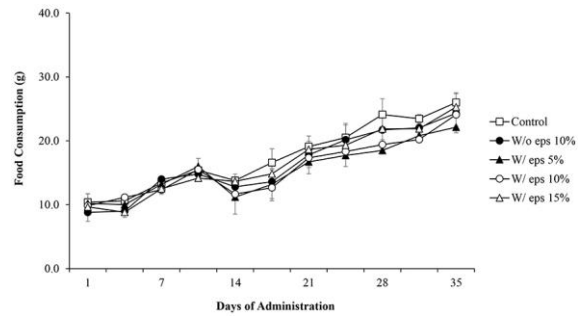
548 **Fig. 2. Mean body weight of Sprague-Dawley (SD) rats orally administered with TMlp**

549 **for 5 weeks.** Values are presented as mean±SD (n=5). TMlp, *Tenebrio molitor* larva powder;

550 W/ eps, expanded-polystyrene-fed; W/o eps, without expanded-polystyrene-fed.

551

ACCEPTED



552

553 **Fig. 3. Food consumption of Sprague-Dawley (SD) rats orally administered TMlp for 5**

554 **weeks.** Values are presented as mean±SD (n=5). TMlp, *Tenebrio molitor* larva powder; W/

555 eps, expanded-polystyrene-fed; W/o eps, without expanded-polystyrene-fed.

556

ACCEPTED

557 **Table 2. Absolute and relative organ weights of Sprague-Dawley (SD) rats orally**
 558 **administered TMlp for 5 weeks.**

Parameters	Control	TMlp			
		W/o eps 10%	W/ eps 5%	W/ eps 10%	W/ eps 15%
Absolute organ weights (g)					
Liver	12.4±1.03	11.96±1.83	11.36±1.51	11.94±1.10	11.87±1.23
Kidney	1.27±0.07	1.22±0.11	1.171±0.13	1.16±0.09	1.20±0.08
Adrenal gland (mg)	26.38±3.96	25.82±2.09	25.09±3.43	23.43±6.51	25.66±3.81
Heart	1.16±0.07	1.23±0.09	1.10±0.01	1.17±0.02	1.23±0.08
Thymus	0.53±0.09	0.57±0.11	0.80±0.28	0.64±0.12	0.71±0.06
Testes	1.92±0.12	1.77±0.07*	1.73±0.12	1.75±0.09	1.83±0.15
Lung	1.49±0.09	1.36±0.16	1.50±0.03	1.46±0.19	1.47±0.13
Spleen	0.82±0.06	0.67±0.07*	0.72±0.07	0.73±0.08	0.79±0.13
Relative organ weights (g%)					
Liver	3.54±0.34	3.57±0.31	3.66±0.73	3.64±0.14	3.48±0.25
Kidney	0.36±0.02	0.37±0.02	0.38±0.06	0.36±0.02	0.35±0.02
Adrenal gland (mg%)	7.38±1.29	7.74±0.21	7.50±0.89	7.30±2.30	7.55±1.16
Heart	0.33±0.03	0.37±0.02	0.35±0.05	0.36±0.02	0.36±0.01
Thymus	0.15±0.03	0.17±0.04	0.26±0.09	0.20±0.04	0.21±0.02
Testes	0.55±0.03	0.53±0.04	0.54±0.09	0.54±0.03	0.54±0.04
Lung	0.42±0.04	0.41±0.03	0.48±0.07	0.45±0.05	0.43±0.03
Spleen	0.24±0.02	0.20±0.02*	0.23±0.03	0.22±0.01	0.23±0.04

559 Values are presented as mean±SD (n=5). Significance was determined using Student's *t*-test;

560 **p*<0.05 compared with control *versus* W/o eps 10%; #*p*<0.05 compared with W/o eps 10%

561 *versus* W/ eps 10%. TMlp, *Tenebrio molitor* larva powder; W/ eps, expanded-polystyrene-fed;

562 W/o eps, without expanded-polystyrene-fed.

563

564

565 **Table 3. Hematological parameters of Sprague-Dawley (SD) rats orally administered**
 566 **TMlp for 5 weeks.**

Parameters	Control	TMlp			
		W/o eps 10%	W/ eps 5%	W/ eps 10%	W/ eps 15%
WBC (K/ μ L)	6.99 \pm 3.12	6.75 \pm 2.05	8.38 \pm 1.60	6.90 \pm 1.54	8.47 \pm 2.07
Neutrophils (%)	11.32 \pm 4.91	6.48 \pm 1.53	7.22 \pm 1.59	14.85 \pm 3.21 [#]	8.20 \pm 1.83
Lymphocyte (%)	83.30 \pm 5.05	89.34 \pm 1.66	89.20 \pm 2.74	79.33 \pm 2.07 [#]	87.35 \pm 2.27
Monocytes (%)	4.36 \pm 0.97	3.00 \pm 1.82	2.72 \pm 1.08	4.00 \pm 0.80	3.17 \pm 1.01
Eosinophils (%)	0.76 \pm 0.27	0.94 \pm 0.35	0.68 \pm 0.29	1.60 \pm 1.17	1.10 \pm 0.25
Basophils (%)	0.26 \pm 0.08	0.24 \pm 0.05	0.24 \pm 0.04	0.22 \pm 0.07	0.18 \pm 0.07
RBC (M/ μ L)	8.59 \pm 0.31	8.79 \pm 0.17	8.74 \pm 0.15	8.51 \pm 0.31	8.26 \pm 0.21
Hemoglobin (g/dL)	15.64 \pm 0.55	15.98 \pm 0.46	15.82 \pm 0.23	15.65 \pm 0.35	15.37 \pm 0.21
Hematocrit (%)	45.28 \pm 1.65	45.82 \pm 1.02	45.90 \pm 0.59	45.02 \pm 1.09	44.15 \pm 0.54
MCV (fL)	52.72 \pm 0.28	52.12 \pm 0.65	52.52 \pm 0.87	52.93 \pm 0.90	53.48 \pm 0.86
MCH (pg)	18.22 \pm 0.13	18.18 \pm 0.34	18.12 \pm 0.40	18.40 \pm 0.44	18.60 \pm 0.34
MCHC (g/dL)	35.54 \pm 0.22	34.88 \pm 0.36	34.48 \pm 0.18	34.77 \pm 0.20	34.82 \pm 0.16
Platelet (K/ μ L)	759.60 \pm 205.57	566.60 \pm 278.75	740.20 \pm 140.80	777.33 \pm 195.51	877.00 \pm 81.06
RDW (%)	13.28 \pm 0.88	12.78 \pm 0.40	13.02 \pm 0.59	12.43 \pm 0.54	11.67 \pm 0.21
MPV (%)	7.24 \pm 0.28	7.58 \pm 0.60	7.30 \pm 0.14	7.27 \pm 0.26	7.23 \pm 0.15

567 Values are presented as mean \pm SD (n=5). Significance was determined using Student's *t*-test;

568 **p*<0.05 compared with control versus W/o eps 10%; #*p*<0.05 compared with W/o eps 10%
 569 versus W/ eps 10%.

570 TMlp, *Tenebrio molitor* larva powder; W/ eps, expanded-polystyrene-fed; W/o eps, without
 571 expanded-polystyrene-fed; WBC, white blood cell; RBC, red blood cell; MCV, mean
 572 corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean cell hemoglobin
 573 concentration; RDW, red cell distribution width; MPV, mean platelet volume.

574

575

576 **Table 4. Serum biochemical parameters of Sprague-Dawley (SD) rats orally**
 577 **administered TMlp for 5 weeks.**

Parameters	Control	TMlp			
		W/o eps 10%	W/ eps 5%	W/ eps 10%	W/ eps 15%
TP (g/dL)	5.74 ± 0.26	5.78 ± 0.19	5.90 ± 0.21	5.95 ± 0.21	5.93 ± 0.17
Albumin (g/dL)	3.56 ± 0.15	3.64 ± 0.14	3.67 ± 0.10	3.77 ± 0.11	3.70 ± 0.06
T-Bili (mg/dL)	LOD ¹⁾	0.0043 ± 0.0086	LOD	LOD	0.0167 ± 0.0373
Glucose (mg/dL)	224.80 ± 7.17	222.40 ± 19.44	212.80 ± 16.12	204.50 ± 17.64	212.83 ± 19.92
BUN (mg/dL)	15.84 ± 0.51	15.04 ± 2.37	15.38 ± 1.23	14.95 ± 1.89	13.85 ± 1.85
Crea (mg/dL)	0.26 ± 0.05	0.26 ± 0.05	0.26 ± 0.05	0.27 ± 0.05	0.25 ± 0.05
AST (U/L)	123.20 ± 32.76	95.20 ± 27.95	104.60 ± 22.22	95.17 ± 20.91	80.50 ± 6.24
ALT (U/L)	61.93 ± 5.11	57.98 ± 5.87	56.17 ± 2.27	59.61 ± 2.92	56.47 ± 5.19
ALP (U/L)	332.80 ± 56.60	334.40 ± 31.93	294.60 ± 46.77	308.83 ± 13.86	299.00 ± 39.49
γ-GTP (U/L)	0.96 ± 0.33	1.05 ± 0.32	1.04 ± 0.36	0.97 ± 0.23	0.77 ± 0.17
Globulin (g/dL)	2.18 ± 0.12	2.14 ± 0.08	2.22 ± 0.12	2.18 ± 0.13	2.23 ± 0.18
Na ⁺ (mmol/L)	138.32 ± 0.52	139.20 ± 0.51*	139.98 ± 1.32	139.98 ± 0.53 [#]	140.00 ± 0.49
K ⁺ (mmol/L)	4.41 ± 0.30	4.00 ± 0.31	4.09 ± 0.34	4.45 ± 0.57	4.23 ± 0.30
Cl ⁻ (mmol/L)	98.20 ± 0.35	99.02 ± 0.63	99.04 ± 1.16	99.15 ± 1.13	98.70 ± 1.03

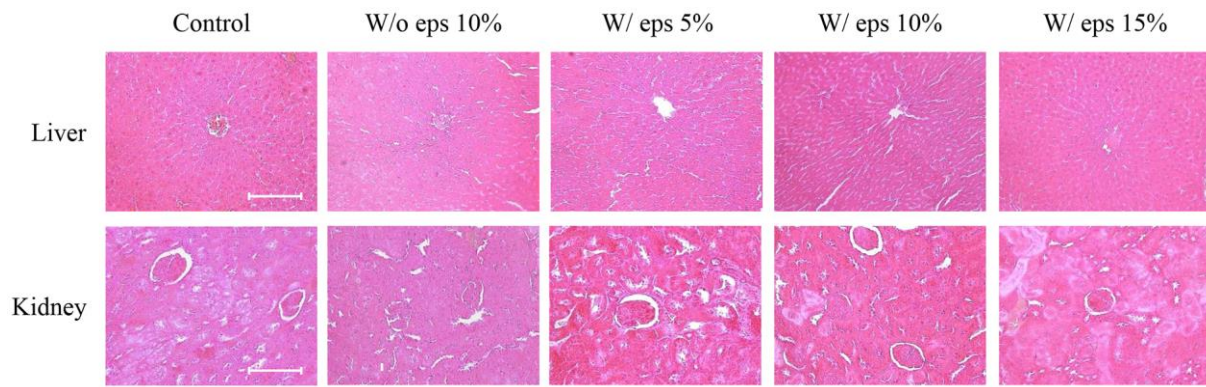
578 1) LOD: Below the limit of detection.

579 Values are presented as mean±SD (n=5). Significance was determined using Student's *t*-test;

580 **p*<0.05 compared with control *versus* W/o eps 10%; [#]*p*<0.05 compared with W/o eps 10%
 581 *versus* W/ eps 10%.

582 TMlp, *Tenebrio molitor* larva powder; W/ eps, expanded-polystyrene-fed; W/o eps, without
 583 expanded-polystyrene-fed; TP, total protein; T-Bili, total bilirubin; BUN, blood urea nitrogen;
 584 Crea, creatinine; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP,
 585 alkaline phosphatase; γ-GTP, gamma glutamyl transpeptidase.

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587

588 **Fig. 4. Histopathological toxicity analysis of the liver and kidney in Sprague-Dawley**
 589 **(SD) rats orally administered TMlp for 5 weeks.** Slides were observed under a light
 590 microscope (scale bar, 10 μ m). TMlp, *Tenebrio molitor* larva powder; W/ eps, expanded-
 591 polystyrene-fed; W/o eps, without expanded-polystyrene-fed.

ACCEPTED