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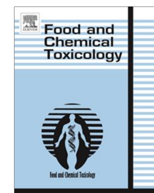

Evaluation of the toxicological safety of erinacine A-enriched *Hericium erinaceus* in a 28-day oral feeding study in Sprague–Dawley rats

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ABSTRACT

Natural products have attained great importance as they are believed to be the new alternative medicines for conventional therapy. As numerous studies have proved the tremendous medicinal values of *Hericium erinaceus*, it is necessary to take into account its safety as well as its risk for the recipient. However, mushroom mycelium has an identity distinct from mushrooms, as two specific classes of compounds, hericenones and erinacines, can only be extracted from both the fruit body and the cultured mycelium, respectively. Therefore, this is the first report on the evaluation of the toxicity of *H. erinaceus* mycelium, enriched with 5 mg/g erinacine A, by a 28-day repeated oral administration study in Sprague–Dawley rats. Three doses of 1 (Low), 2 (Mid) and 3 (High) g/kg body weight/day were selected for the study while distilled water served as control. All animals survived to the end of the study. No abnormal changes were observed in clinical signs. No adverse or test article-related differences were found in urinalysis, haematology and serum biochemistry parameters, between the treatment and control groups. No gross pathological findings and histopathological differences were seen. Therefore, the no-observed-adverse-effect level of erinacine A-enriched *H. erinaceus* is greater than 3 g/kg body weight/day.

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1. Introduction

Hericium erinaceus (*H. erinaceus*), the ideal culinary-medicinal mushroom, also known as *monkey head mushroom* in Chinese or *yamabushitake* in Japanese, has been reported widely to possess anti-cancer, anti-microbial, anti-diabetic, anti-hypertensive, anti-fat deposit, antioxidant, gastro-protective, neuro-protective, immuno-modulating, and wound-healing properties (Abdulla et al., 2011; Hiwatashi et al., 2010; Kim et al., 2011, 2012a, 2012b, 2014; Shang et al., 2013; Wong et al., 2011, 2013; Zhang et al., 2012). Among these potent health-enhancing functions of *H. erinaceus*, recent research focused on its most sought after characteristic out of all other edible mushrooms with medicinal values, which is its protecting and stimulating effects on nerve cells. Hericenones from C to H (Kawagishi et al., 1991, 1992) and erinacines from A to I (Kawagishi et al., 1994, 1996a, 1996b; Lee et al., 2000), isolated from the methanol extract of the fruit body and cultured mycelium of *H. erinaceus*, respectively (Ma et al., 2010), were all shown to be powerful stimulators of nerve growth factor (NGF) biosynthesis in cultured rodent astrocytes. Hericenones C, D and E were reported to be stimulators of NGF biosynthesis in mouse astroglial cells at 33 mg/mL (Kawagishi et al., 1991) but they did not increase NGF mRNA expression at 10–100 mg/mL in 1321N1 human astrocytoma cells and failed to stimulate NGF mRNA expression in primary cultured rat astroglial cells (Mori et al., 2008). While there are still debates about whether hericenones stimulate NGF synthesis, erinacine A, on the other hand, demonstrated its profound NGF-inducing activities *in vitro* and *in vivo* (Shimbo et al., 2005). Such NGF stimulating effects were augmented by the increased availability of the active compound and

Abbreviations: EAHE, erinacine A-enriched *H. erinaceus*; HPLC, high performance liquid chromatography; NGF, nerve growth factor; S–D, Sprague–Dawley; SD, standard deviation.

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moreover, achieved by oral administration (Mori et al., 2009). The induction of NGF is critical for proper neural growth, neurite outgrowth (Madduri et al., 2009), survival (Freeman et al., 2004) and maintenance (Lorz and Mehmet, 2009; Skaper, 2008). Furthermore, NGF levels lower than normal have been shown to be linked to early stages of neurological diseases (Covaceuszach et al., 2009; Giacobini and Becker, 2007; Schulte-Herbruggen et al., 2007, 2008). Hence, there is potential in developing *H. erinaceus* enriched with erinacine A as an ingredient in functional foods or medicinal products intended to ameliorate neurodegenerative symptoms.

The molecular composition within the fruiting bodies of *H. erinaceus*, unfortunately, varied significantly among environmental conditions. The cultivation is time-consuming and most notably, the fruiting body was reported to contain no erinacine. Therefore, it seems highly desirable to produce *H. erinaceus* by submerged fermentation in a batch stirred tank bioreactor with constantly controlled culture parameters for achieving both constant mycelia composition and bioactive metabolites, which would be useful for further development on an industrial scale. Under optimal conditions, the production of erinacine A was 192 ± 42 mg/L in a 10-L bioreactor (Krzyczkowski et al., 2010).

The use of erinacine A as an ingredient in functional foods or medicinal products has attracted considerable attention as a consequence of attempts to reduce the risk of getting neurodegenerative diseases in Asian countries (Kawagishi and Zhuang, 2008; Kim et al., 2013; Xu et al., 2014). A previous study had shown that intragastric dosing erinacine A at 8 mg/kg body weight (BW) increased NGF expression in the central nervous systems of rats (Shimbo et al., 2005). An experiment with orally administrated erinacine A-enriched *H. erinaceus* (EAHE) mycelium revealed its potential in reducing occlusion-induced brain infarction in rats (unpublished results). The mushroom mycelium is regarded as suitable for food use and has been approved by Taiwan Food and Drug Administration (TFDA, 2010). Hence, the present study was undertaken to determine the sub-acute oral dosing of EAHE mycelium in experimental animals to provide information on the safety of this new promising natural remedy.

2. Materials and methods

2.1. Sample preparation

H. erinaceus (BCRC 35669) purchased from Bioresources Collection and Research Center (BCRC) in Food Industry Research and Development Institute (Hsinchu, Taiwan) was maintained on potato dextrose agar at 26 °C for 15 days. After incubation, a mycelial agar block (1 cm^3) was removed, transferred to a 2-L Erlenmeyer flask containing 1.3 L synthetic medium (composed of 0.25% yeast extract, 4.5% glucose, 0.5% soybean powder, 0.25% peptone and 0.05% $MgSO_4$, adjusted to pH 4.5) and incubated for 5 days at 26 °C on a rotary shaker (120 rpm). The fermentation process was then scaled up from a 2-L shake flask to 500-L and 20-ton fermenters for 5 days and 12 days, respectively. At the end of the fermentation process, the mycelia were then harvested, lyophilized, grounded into powder, and stored in a desiccator at room temperature. The proximate composition analysis, including ash, total protein, lipids, and carbohydrates of the freeze-dried mycelia were determined according to official AOAC methods (International, 1995). The dry sample was then analyzed by high performance liquid chromatography (HPLC) to evaluate the active erinacine A content in the mycelia based on its dry weight. The *H. erinaceus* freeze-dried mycelium powder containing 5 mg/g erinacine A was dissolved in distilled water with vigorous shaking in order to get a final concentration of 50, 100 and 150 mg/mL before its administration to Sprague–Dawley (S–D) rats. Different concentrations were prepared for a constant volume of 20 mL/kg BW/day at all dose levels to prevent the adverse effect of gavage.

2.2. Identification of erinacine A

The identification of erinacine A in *H. erinaceus* extract using nuclear magnetic resonance (NMR) and liquid chromatography–mass spectrometry (LC–MSMS) have been previously described (Krzyczkowski et al., 2009, 2010). NMR spectra recorded at 500 MHz for ^1H and 125 MHz for ^{13}C were established in $CDCl_3$ as an internal standard on a Varian Unity INOVA-500 spectrometer. LC–MSMS analysis were carried out on erinacine A by positive mode electrospray ionization method on API 3000 (Applied Biosystem, Foster City, CA, USA) triple quadrupole mass spectrometer attached to Agilent 1100 HPLC (Agilent Technologies, Santa Clara, CA). HPLC analysis of erinacine A was executed according to the previous study (Krzyczkowski et al., 2010) with minor modifications. The analytical column used was a COSMOSIL 5C18-AR-II (250 × 4.6 mm; particle size 5 μm, Nacalai USA, Inc.). Separation was performed at 40 °C using two different gradients for the mobile phase, which is consisted of two solvents, methanol (A) and 2.0% acetic acid in water (B). The gradient elution had the following profile: 0–20 min, 60–90% (A); 20–25 min, 90% (A). Retention time of erinacine A was approximately ~17 min at a flow rate of 1.0 mL/min with a scanning UV wavelength at 340 nm.

2.3. Animals

Forty male and 40 female S–D rats obtained from BioLASCO Taiwan Co., Ltd. were acclimated and quarantined for 1 week prior to the initiation of the study. The animals were housed in pairs in polypropylene cages with absorbent hardwood bedding (Beta Chip; Northeastern Products Corp, USA). The rats were maintained in a well-ventilated room (10–15 air changes/h) under an ambient temperature of 22 ± 3 °C and 55 ± 15% relative humidity, with a 12:12 h light regime. The animals were provided with standard rodent diet (MGF; Oriental Yeast Co., Ltd., Tokyo, Japan) and purified water *ad libitum*. Before the start of the study, the animals were 7–8 weeks of age and had their health status evaluated. The study was approved by the Institutional Animal Care and Use Committee (IACUC No. 101-9c).

2.4. Study design

The entire study was conducted in accordance to Organization for Economic Cooperation and Development (OECD, 2001) Guideline 407 and in compliance with recent Good Laboratory Practice regulations. All young healthy adult S–D rats were randomly divided into three treatment groups and a control group, each consisting of ten male and female rats, according to their body mass. Our earlier study on acute oral toxicity indicated that acute oral LD_{50} of EAHE was greater than 5 g/kg in S–D rats. Hence, three doses of 1 g/kg (Low dose), 2 g/kg (Mid dose) and 3 g/kg (High dose) were selected for the study, whereas distilled water was served as the control. The body weight of all rats was recorded at baseline and then was noted weekly until their scheduled necropsy. Average measurement of water and food intake (the difference between the food given and their remnants on the next day) was conducted weekly during the study period for both male and female rats. Fresh 20 mL/kg EAHE was given daily by gavage using a straight, stainless steel ball-tipped feeding needle for 28 consecutive days. All experimental animals were observed once a day for signs of toxicity, mortality and morbidity till the completion of treatment. An ophthalmological examination with naked eyes and indirect ophthalmoscopy was performed on all animals prior to initiation of dosing and before sacrifice. At the end of the stipulated experimental period, all overnight fasted (water allowed) animals were anesthetized with carbon dioxide and euthanized after blood samples were collected via the intracardiac puncture method into EDTA-containing, sodium citrate-containing (both for haematological analysis) and non-anticoagulate-containing tubes (for biochemistry analysis).

2.5. Toxicological evaluations

Blood samples and urine were collected from the animals before they were sacrificed. Blood samples were analyzed for haematology, coagulation time and serum biochemistry values. The haematological parameters including haematocrit, haemoglobin, red blood cell (RBC), white blood cell (WBC), platelet count, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), lymphocyte, neutrophil, monocyte, eosinophil, basophil and reticulocyte were measured using an automated haematology analyzer (Gen-S™, Beckman, Coulter, USA). The coagulation parameter such as prothrombin time (PT) was performed using a fully automated blood coagulation analyzer (Sysmex CA-1500, Kobe, Japan) on blood collected with citrate. For serum biochemistry analysis, the serum was separated from blood after centrifugation and examined using an automated biochemistry analyzer (Beckman LX®-20, California, USA), which includes analysis of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, total protein, total bilirubin, creatinine, blood urea nitrogen (BUN), glucose, cholesterol, triglyceride, phosphorus, calcium, chloride, potassium and sodium.

Sixteen-hour urine specimens were evaluated using a semiquantitative urinalysis system (Urisys 2400, Roche, Basel, Switzerland) for the following parameters: specific gravity (SG), color, protein, urobilinogen, pH, ketone, bilirubin, glucose, nitrite, and occult blood. Furthermore, a microscopic examination of urine sediments was used to observe the presence of WBC, RBC, epithelial cell (EP), crystals, and microbes.

2.6. Pathology and histopathology

After blood collection, all animals were sacrificed by exsanguination followed by a full, detailed gross pathological examination of the external surfaces of the body, all orifices, the cranial, thoracic and abdominal cavities, and their contents

in situ. Organs such as the brain, heart, kidney, liver, spleen, adrenal gland, and testis/ovary were trimmed free of fat and weighted. Absolute and relative organ weights (Relative organ weight (%) = organ weight (g)/body weight (g) × 100) were calculated. These organs obtained from the animals of the control and high dose groups were then retained for histopathology. Except for testes, which were initially fixed in modified Davidson's solution and then transferred to 10% neutral buffered formalin after 24 h, other organs were fixed solely in 10% neutral buffered formalin. Fixed-organs were processed to produce 5 μm paraffin wax tissue sections, stained with hematoxylin and eosin and then microscopically examined (Nikon Opticphot-2, Tokyo, Japan). If treatment-related changes were observed in the high dose group, the low and mid dose groups were to be investigated.

2.7. Statistical analysis

All values presented throughout this manuscript were in mean ± standard deviation (SD). Mean difference between the control and the treatment groups were analyzed by one-way ANOVA followed by Duncan's test. A value of $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

Natural products have attained great importance as they are believed to be the new alternative medicines for conventional therapy. As numerous studies have proved the tremendous medicinal values of *H. erinaceus* (Khan et al., 2013), it is necessary to take into account its safety as well as its risk for the recipient. However, mushroom mycelium has an identity distinct from mushrooms, as two specific classes of compounds, hericenones and erinacines, can only be extracted from both the fruit body and the cultured mycelium, respectively. Therefore, this is the first report on the evaluation of the toxicity of *H. erinaceus* mycelium, enriched with 5 mg/g erinacine A (EAHE), by a 28-day repeated oral administration study in rats.

The proximate analysis of freeze-dried EAHE mycelium showed 5.5% moisture, 10.5% ash, 25.8% crude protein, 9.5% crude fat and 48.7% carbohydrate. The moisture, ash, and crude protein levels obtained were comparable to the values reported earlier (Li et al., 2013) (4.80%, 9.01% and 25.71%, respectively). The crude fat and carbohydrate contents were higher than previously reported (3.44% and 25.65%, respectively). Such results may be attributed to the different medium selected in this study, which is to cultivate *H. erinaceus* mycelium with enriched erinacine A.

The isolation and identification of erinacine A in *H. erinaceus* extract have been previously described (Krzyczkowski et al., 2009, 2010). Erinacine A was identified by the $[M+H]^+$ ion peak in LC–MSMS at m/z 433 (required m/z 432) and after being concentrated, it was subjected to column chromatography on silica gel. The ^1H, ^{13}C NMR spectroscopy (not shown) further verified the structure of erinacine A and matched the published data in a consistent manner (Krzyczkowski et al., 2010). The 5 mg/g erinacine A in the *H. erinaceus* extracted with 85% ethanol was confirmed and quantified by HPLC as shown in Fig. 1.

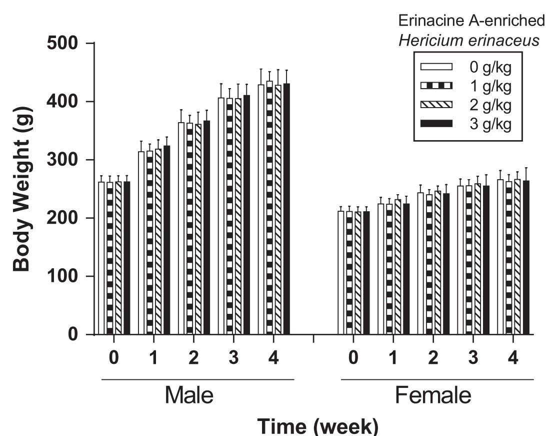

Fig. 2. Effect of EAHE on body weight gain in male and female S–D rats during the 28-day safety assessment. Data were expressed as mean ± SD (n = 10).

Daily oral administration of EAHE at all tested doses (1, 2 and 3 g/kg) for a period of 28 days did not induce any symptoms of toxicity, morbidity or mortality in both sexes of rats (n = 80). Additionally, no ophthalmic abnormalities were noted in any of the animals before and after the experiment (data not shown). Regarding body weight, no significant difference was observed between the control and treated groups ($p > 0.05$, one-way ANOVA followed by Duncan's test) (Fig. 2). Feed intake of EAHE in all groups was not statistically different from those of the control group except for the female rats in the high dose group, which consumed less (15.6 ± 1.0) during the first week of the study ($p < 0.05$, one-way ANOVA followed by Duncan's test) (Fig. 3).

Haematological analysis revealed a significantly higher value of prothrombin time (PT) (11.6 ± 0.7) in males of the high dose group and a significantly lower value of reticulocyte (2.4 ± 0.7) in females of the mid dose group, as compared to the control group (Male PT: (10.6 ± 0.7); Female reticulocyte: (3.4 ± 1.1); $p < 0.05$, one-way ANOVA followed by Duncan's test) (Table 1). However, such values were found to be within the normal range of rats (Levine, 2002), thus indicating the result of normal variation among animal groups. Other parameters such as haematocrit, haemoglobin, red blood cell, white blood cell, platelet count, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, lymphocyte, neutrophil, monocyte,

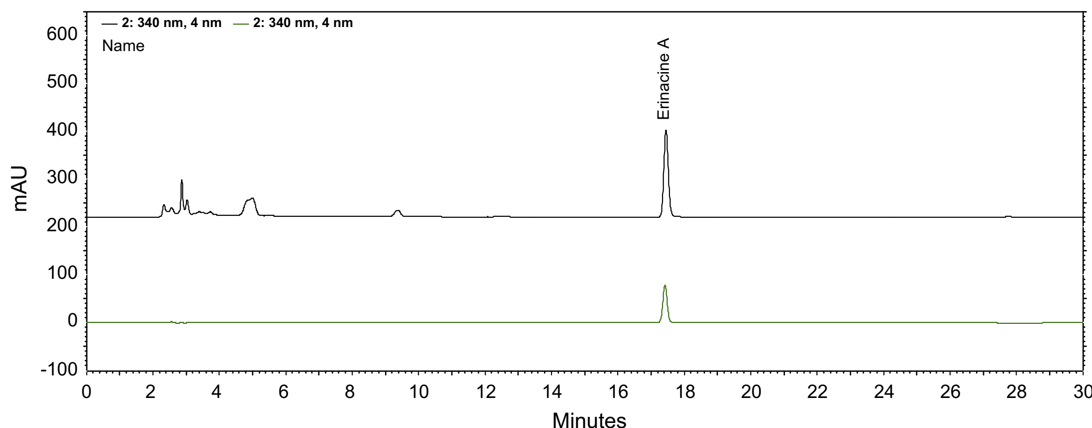

Fig. 1. The HPLC chromatograms of erinaince A standard sample (bottom) and the ethanol extract of *H. erinaceus* mycelium (top) from 20-ton bioreactor (UV detection at 340 nm). Retention time of erinacine A is 17.4 min.

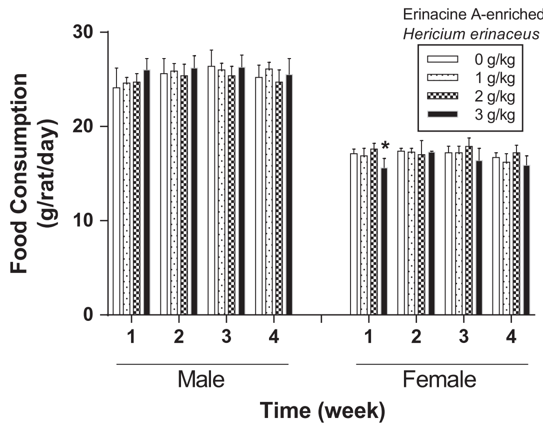

Fig. 3. Effect of EAHE on feed intake in male and female S–D rats during the 28-day safety assessment. Data were expressed as mean ± SD ($n = 5$ cage, two animals per cage). *Statistically significant ($p < 0.05$) when compared to the control group.

eosinophil, and basophil showed no significant difference when compared to the control group ($p > 0.05$, one-way ANOVA followed by Duncan's test). Serum biochemistry analysis revealed that globulin levels were statistically higher (2.7 ± 0.3) in male rats treated with 2 g/kg EAHE when compared to the control group (2.5 ± 0.2) ($p < 0.05$, one-way ANOVA followed by Duncan's test) (Table 2).

However, such values were in the range of normal values (Levine, 2002), which suggested normal function of the organs. No other significant differences in serum biochemistry parameters were observed among the groups. In urinalysis, there were no significant changes between any of the EAHE treated groups and the control group (data not shown).

The absolute weight of brain, heart, kidney, liver, spleen, adrenal gland, and testis/ovary recorded among groups were found with no significant difference ($p > 0.05$, one-way ANOVA followed by Duncan's test). The only exception was observed in the absolute weight of the kidney in the male rats of the high dose group (data not shown). Hence, it was found that the relative weights of testis (0.839 ± 0.068) and relative weights of testis and kidney (0.823 ± 0.072 and 0.835 ± 0.067) in male rats treated with 2 and 3 g/kg EAHE, respectively, were higher than the control group (testis: 0.756 ± 0.086; kidney: 0.755 ± 0.028). Nonetheless, all of the increases were non-dose dependent, and the differences may have been due to the variation in size of internal organs and/or body weight of the animals (Levine, 2002). For female rats, the relative weight of the adrenal glands (0.031 ± 0.005) in the high dose group increased as compared with the control group (0.027 ± 0.002) while the other treatment groups showed no significant difference ($p > 0.05$, one-way ANOVA followed by Duncan's test) (Table 3). Necropsy and histopathology examinations were further conducted to confirm whether or not the organs or tissue of the adrenal glands had been damaged and showed no significant changes compared to those of the control group ($p > 0.05$, one-way ANOVA followed by Duncan's test). In summary, no treatment-related findings were noted.

Upon histopathological examination (Shackelford et al., 2002) in control and high dose groups, no treatment-related toxicological

Table 1
Effects of EAHE on haematological parameters in male and female S–D rats during the 28-day safety assessment.

Parameters	Control (distilled water)	Erinacine A-enriched *H. erinaceus* (g/kg)		
		1 g/kg	2 g/kg	3 g/kg
Male				
WBC (10^3/μl)	13.5 ± 2.4	13.6 ± 2.6	11.5 ± 2.9	11.6 ± 3.8
RBC (10^6/μl)	8.4 ± 0.4	8.2 ± 0.5	8.3 ± 0.6	8.3 ± 0.3
Haemoglobin (g/dL)	16.5 ± 0.9	16.2 ± 0.7	16.6 ± 1.2	16.6 ± 0.5
Haematocrit (%)	51.7 ± 2.8	51.7 ± 2.5	52.0 ± 3.7	51.9 ± 2.3
MCV (fL)	61.4 ± 0.9	62.9 ± 3.1	62.6 ± 2.7	62.6 ± 1.9
MCH (pg)	19.5 ± 0.5	19.8 ± 0.7	20.0 ± 0.5	20.0 ± 0.6
MCHC (g/dL)	31.8 ± 0.9	31.4 ± 1.1	31.9 ± 1.0	32.0 ± 0.9
Platelet (10^3/μl)	709.6 ± 92.0	697.8 ± 151.4	734.1 ± 113.4	703.5 ± 76.0
Neutrophil (%)	10.9 ± 2.5	10.8 ± 3.9	12.9 ± 5.5	13.5 ± 4.9
Lymphocyte (%)	86.0 ± 2.1	85.7 ± 5.0	84.4 ± 5.6	83.3 ± 4.8
Monocyte (%)	2.1 ± 0.7	2.3 ± 0.9	1.9 ± 1.1	2.4 ± 0.7
Eosinophil (%)	0.9 ± 0.2	1.1 ± 0.9	0.8 ± 0.4	0.8 ± 0.2
Basophil (%)	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
Reticulocyte (%)	2.0 ± 0.7	1.6 ± 0.4	1.9 ± 0.4	1.9 ± 0.4
PT (s)	10.6 ± 0.7	10.8 ± 0.5	11.1 ± 0.4	11.6 ± 0.7*
Female				
WBC (10^3/μl)	9.0 ± 5.0	10.8 ± 4.8	10.4 ± 3.7	11.3 ± 3.6
RBC (10^6/μl)	8.2 ± 0.3	7.9 ± 0.7	8.1 ± 0.4	8.1 ± 0.5
Haemoglobin (g/dL)	15.9 ± 0.7	15.9 ± 0.8	16.3 ± 0.9	15.8 ± 0.6
Haematocrit (%)	51.3 ± 3.0	49.8 ± 4.7	51.7 ± 2.3	49.6 ± 2.1
MCV (fL)	62.7 ± 3.3	62.8 ± 3.5	63.9 ± 2.2	61.3 ± 3.0
MCH (pg)	19.4 ± 0.7	20.1 ± 1.2	20.1 ± 0.3	19.6 ± 1.0
MCHC (g/dL)	30.9 ± 1.0	32.1 ± 2.2	31.5 ± 1.0	31.9 ± 0.6
Platelet (10^3/μl)	681.2 ± 113.7	801.6 ± 172.8	737.8 ± 98.3	799.9 ± 111.6
Neutrophil (%)	12.9 ± 1.9	22.2 ± 13.0	17.4 ± 8.9	14.7 ± 4.6
Lymphocyte (%)	82.6 ± 1.9	73.1 ± 13.2	75.6 ± 14.4	80.8 ± 4.9
Monocyte (%)	3.2 ± 1.3	3.1 ± 1.1	5.7 ± 6.4	3.3 ± 1.0
Eosinophil (%)	1.2 ± 0.4	1.4 ± 0.7	1.1 ± 0.6	1.0 ± 0.5
Basophil (%)	0.2 ± 0.3	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Reticulocyte (%)	3.4 ± 1.1	3.6 ± 1.1	2.4 ± 0.7*	2.7 ± 0.9
PT (s)	9.9 ± 0.2	10.0 ± 0.2	10.0 ± 0.2	10.0 ± 0.3

Data were expressed as mean ± SD ($n = 10$).
White blood cell (WBC); red blood cell (RBC); mean corpuscular volume (MCV); mean corpuscular haemoglobin (MCH); mean corpuscular haemoglobin concentration (MCHC); prothrombin time (PT).
* Statistically significant ($p < 0.05$) when compared to the control group.

Table 2
Effects of EAHE on serum biochemistry parameters in male and female S–D rats during the 28-day safety assessment.

Parameters	Control (distilled water)	Erinacine A-enriched *H. erinaceus* (g/kg)		
		1 g/kg	2 g/kg	3 g/kg
Male				
AST (U/L)	108.3 ± 25.8	152.5 ± 83.4	120.9 ± 73.0	132.3 ± 30.3
ALT (U/L)	43.7 ± 8.2	60.2 ± 38.4	60.4 ± 47.5	49.7 ± 10.0
ALP (U/L)	248.3 ± 77.8	199.5 ± 45.1	242.2 ± 99.0	232.5 ± 63.4
Total bilirubin (μg/dL)	32.9 ± 11.3	31.9 ± 11.2	29.0 ± 6.1	31.7 ± 7.2
Total protein (g/dL)	6.7 ± 0.3	6.7 ± 0.3	7.0 ± 0.5	6.8 ± 0.3
Albumin (g/dL)	4.2 ± 0.2	4.2 ± 0.2	4.3 ± 0.3	4.2 ± 0.2
Globulin (g/dL)	2.5 ± 0.2	2.4 ± 0.2	2.7 ± 0.3*	2.6 ± 0.2
BUN (mg/dL)	16.8 ± 2.0	16.4 ± 2.5	18.4 ± 1.9	17.5 ± 2.1
Creatinine (mg/dL)	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.1	0.4 ± 0.0
Glucose (mg/dL)	192.3 ± 34.2	189.6 ± 52.8	198.7 ± 36.3	166.2 ± 56.4
Triglyceride (mg/dL)	85.3 ± 34.4	90.5 ± 40.9	77.7 ± 28.1	84.7 ± 37.2
Cholesterol (mg/dL)	65.6 ± 7.8	69.6 ± 14.1	72.4 ± 16.1	67.2 ± 8.7
Sodium (meq/L)	152.7 ± 3.5	152.1 ± 3.2	152.8 ± 2.9	152.1 ± 2.4
Potassium (meq/L)	6.0 ± 0.4	6.7 ± 0.8	6.4 ± 1.1	6.6 ± 1.0
Calcium (mg/dL)	12.3 ± 0.4	12.4 ± 0.5	12.3 ± 0.3	12.4 ± 0.5
Chloride (meq/L)	102.1 ± 2.8	101.4 ± 2.8	102.3 ± 3.2	100.9 ± 2.4
Phosphorus (mg/dL)	11.3 ± 0.9	12.3 ± 0.9	11.7 ± 2.1	12.6 ± 1.6
Female				
AST (U/L)	156.5 ± 55.4	146.9 ± 64.1	151.6 ± 64.9	131.8 ± 39.0
ALT (U/L)	39.2 ± 19.4	39.8 ± 30.2	41.6 ± 29.2	31.7 ± 11.0
ALP (U/L)	102.8 ± 22.0	90.2 ± 17.7	119.2 ± 44.3	90.7 ± 27.2
Total bilirubin (μg/dL)	36.1 ± 8.4	35.3 ± 18.0	32.6 ± 12.5	35.9 ± 11.5
Total protein (g/dL)	7.1 ± 0.4	7.5 ± 0.5	7.3 ± 0.4	7.2 ± 0.3
Albumin (g/dL)	4.5 ± 0.1	4.7 ± 0.3	4.6 ± 0.2	4.5 ± 0.2
Globulin (g/dL)	2.6 ± 0.3	2.8 ± 0.3	2.7 ± 0.1	2.6 ± 0.2
BUN (mg/dL)	17.2 ± 1.5	16.6 ± 3.0	18.1 ± 2.6	18.4 ± 3.4
Creatinine (mg/dL)	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
Glucose (mg/dL)	115.9 ± 27.1	126.6 ± 21.1	129.4 ± 29.1	136.2 ± 41.1
Triglyceride (mg/dL)	59.4 ± 13.1	53.0 ± 7.7	59.0 ± 25.2	44.0 ± 9.0
Cholesterol (mg/dL)	85.1 ± 11.9	92.6 ± 19.7	88.6 ± 22.7	81.4 ± 18.3
Sodium (meq/L)	151.4 ± 1.3	151.6 ± 3.3	151.2 ± 1.3	150.9 ± 3.3
Potassium (meq/L)	7.4 ± 0.9	7.2 ± 0.5	6.6 ± 0.4	7.1 ± 0.6
Calcium (mg/dL)	12.2 ± 0.4	12.2 ± 0.4	12.3 ± 0.3	12.5 ± 0.9
Chloride (meq/L)	105.5 ± 2.9	104.6 ± 2.8	102.8 ± 2.9	103.7 ± 1.9
Phosphorus (mg/dL)	12.5 ± 1.0	12.2 ± 1.2	11.8 ± 1.3	12.1 ± 1.3

Data were expressed as mean ± SD ($n = 10$).
Aspartate aminotransferase (AST); alanine aminotransferase (ALT); alkaline phosphatase (ALP); blood urea nitrogen (BUN).
* Statistically significant ($p < 0.05$) when compared to the control group.

Table 3
Effects of EAHE on relative organ weight of male and female S–D rats at the end of the 28-day safety assessment.

Relative weight (%)	Control (distilled water)	Erinacine A-enriched *H. erinaceus* (g/kg)		
		1 g/kg	2 g/kg	3 g/kg
Male				
Testes	0.756 ± 0.086	0.789 ± 0.036	0.839 ± 0.068*	0.823 ± 0.072*
Spleen	0.191 ± 0.017	0.186 ± 0.022	0.202 ± 0.049	0.190 ± 0.024
Kidney	0.755 ± 0.028	0.793 ± 0.092	0.764 ± 0.052	0.835 ± 0.067*
Adrenal glands	0.015 ± 0.002	0.015 ± 0.002	0.015 ± 0.002	0.015 ± 0.002
Liver	3.530 ± 0.560	3.540 ± 0.490	3.450 ± 0.590	3.510 ± 0.570
Heart	0.376 ± 0.033	0.390 ± 0.029	0.383 ± 0.045	0.404 ± 0.018
Brain	0.492 ± 0.020	0.499 ± 0.027	0.500 ± 0.061	0.500 ± 0.031
Female				
Ovary	0.034 ± 0.006	0.035 ± 0.005	0.037 ± 0.006	0.037 ± 0.006
Spleen	0.224 ± 0.031	0.210 ± 0.012	0.245 ± 0.022	0.234 ± 0.024
Kidney	0.789 ± 0.049	0.823 ± 0.050	0.799 ± 0.055	0.778 ± 0.039
Adrenal glands	0.027 ± 0.002	0.027 ± 0.003	0.029 ± 0.003	0.031 ± 0.005*
Liver	3.230 ± 0.120	3.240 ± 0.160	3.320 ± 0.230	3.220 ± 0.160
Heart	0.433 ± 0.104	0.414 ± 0.027	0.402 ± 0.038	0.411 ± 0.044
Brain	0.735 ± 0.126	0.770 ± 0.028	0.765 ± 0.049	0.766 ± 0.062

Data were expressed as mean ± SD ($n = 10$).
* Statistically significant ($p < 0.05$) when compared to the control group.

significances were observed in the brain, heart, kidney, liver, spleen, adrenal gland, and testis/ovary (Fig. 4).

Non-treatment related histopathological changes included rats with focal mononuclear cell infiltration in the heart, focal tubular cast, focal interstitial fibrosis, focal mononuclear cell infiltration and focal tubular regeneration in the kidney and multiple fat droplet infiltration, diffuse glycogen infiltration, and focal mononuclear cell infiltration in the liver (Table 4). These changes were said to be

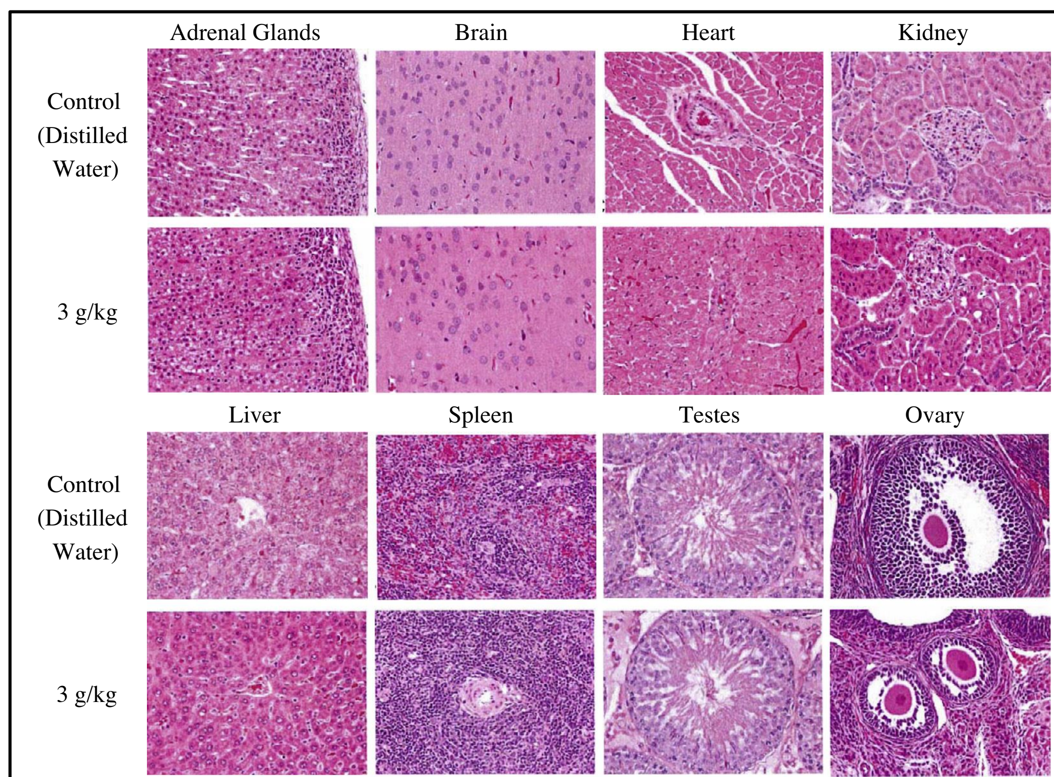

Fig. 4. The histology of organs from the control and high dose groups (400× magnification). No significant damage was detected in the high dose group.

Table 4
Non-treatment related histopathological changes in control and EAHE treated animals for 28 days.

Organ	Lesion	Male		Female	
		Control (distilled water)	3 g/kg	Control (distilled water)	3 g/kg
Heart	Infiltration, mononuclear cell, focal, slight[a]	2/10[b]	–	–	–
Kidney	Cast, tubule, focal, minimal	–	1/10	–	–
	Fibrosis, interstitial, focal, slight	–	1/10	1/10	–
	Infiltration, mononuclear cell, focal, slight	–	1/10	1/10	–
	Regeneration, tubule, focal, slight	–	1/10	1/10	–
Liver	Infiltration, fat droplet, multiple, slight to moderate	3/10	4/10	10/10	10/10
	Infiltration, glycogen, diffuse, slight	6/10	3/10	–	–
	Infiltration, mononuclear cell, focal, slight	2/10	3/10	3/10	2/10

– No effect.
[a] Degree of lesions was graded from one to five depending on severity: minimal (<1%); slight (1–25%); moderate (26–50%); moderate/severe (51–75%); severe/high (76–100%).
[b] Incidence: Affected rats/total examined rats ($n = 10$).

non-treatment related because such alterations were mostly observed in the control group or were comparable among the groups.

4. Conclusion

The 28-day sub-chronic toxicity test in S–D rats showed no evidence of systemic toxicity attributable to EAHE administration. Based on these findings, the no-observed-adverse-effect of EAHE is greater than 3 g/kg BW/day. Additional research on EAHE, including an assessment of its mutagenic and carcinogenic potential, may be included in future studies to further support the safety of its consumption.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

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

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